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# INFLUENCE OF *ESCHERICHIA COLI* ENDOTOXIN ON THE FUNCTIONAL INTEGRITY OF THE GASTROINTESTINAL TRACT OF THE RAT

BY

DANLADI A. AMEH

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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OF DOCTOR OF PHILOSOPHY
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DEPARTMENT OF ANIMAL SCIENCE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **'Influence of *Escherichia coli* Endotoxin on the Functional Integrity of the Gastrointestinal Tract of the Rat'** submitted by **Danladi Amodu Ameh** in partial fulfilment of the requirements for the degree of **Doctor of Philosophy** in **Animal Biochemistry**.

J.R. Thompson

(J.R. Thompson, Supervisor)

for (V.E Baracos)

(A.B.R. Thomson)

Michael I. McBurney

(M.J. McBurney)

(J.B. Meddings, External Examiner)

Date Sept. 24/91

# Abstract

Gastrointestinal tract disturbances which may derive from alterations in metabolism and functional integrity of the gut following exposure to bacterial toxin are quite common in humans and animals. The effects of *E. coli (O55:B5)* endotoxin on the metabolism and functional integrity of the small intestine were studied in the rat. $^{14}CO_2$ production from [U-$^{14}$C]glucose and apparent transport of glucose and amino acids were studied *in vitro* in Ussing chambers. Enterocyte nitrogen metabolism was studied in the presence or absence of glutamine and/or glucose as energy substrates. The consequences of ip injection of two endotoxin levels (100 μg/kg body weight or 400 μg/kg body weight), over a 192 h period, on enterocyte disaccharidase activity and [$^3$H]thymidine incorporation as well as the effects on villi structure, plasma and muscle glutamine concentrations were also examined.

In isolated intestine, apparent glucose, glutamic acid, aspartic acid, serine and threonine transport was decreased ($P<0.05$) by 20 ng/ml endotoxin treatment. Endotoxin treated intestine metabolised a higher proportion of the glucose that it transported than control intestine.

Considerable amounts of glutamate (126.9 ± 11.4 or 121.8 ± 12.7 nmol/h/mg) and alanine (25.3 ± 2.4 or 52.8 ± 4.8 nmol/h/mg) were produced by isolated enterocytes incubated with 5 mM glutamine or 5 mM glutamine plus 10 mM glucose, respectively. Endotoxin (50 ng/ml) increased ($P<0.001$) the rate of [$^3$H]phenylalanine release from prelabelled protein in the presence or absence of energy substrates or plasma concentrations of amino acids but had no effect on tyrosine release which was enhanced in the presence of 5 mM glutamine.

Endotoxin injection increased ($P<0.01$) enterocyte [$^3$H]thymidine

incorporation and caused a dramatic decrease in food intake during the initial endotoxin challenge. Enterocyte maltase, sucrase and isomaltase activities in endotoxin treated rats were not different (P>0.05) from activities observed in the pairfed controls injected with saline. Lactase activity was depressed (P<0.05) by endotoxin treatment only at 400 μg/kg body weight.

Endotoxin administration for 8 d decreased (P<0.05) plasma and gastrocnemius free glutamine concentrations and in addition, decreased (P<0.01) villi height and increased crypt depth. Dietary glutamine supplementation (4%) restored plasma and gastrocnemius free glutamine concentrations following 192 h treatment period. The villi height and crypt depth in the glutamine supplemented endotoxin treated rats were not different (P>0.05) from saline injected controls.

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# I General Introduction

Infections with bacteria and/or viruses continue to be a major cause of death and production losses to man and his domestic animals. In developing countries, acute infectious diarrhea, and systemic infections are leading causes of mortality in children and in domestic animals (Snyder and Merson, 1982). Enterotoxigenic *Escherichia coli* (*E. coli*) and rotaviruses have been implicated by numerous human and veterinary surveys as the two most common etiological agents of infectious diarrhea (Tzipori et al., 1982; Newsome and Coney, 1985).

*Escherichia coli* is a Gram-negative rod shaped bacterium belonging to the family enterobacteriaceae and is the only member of the genus *Escherichia*. It is a facultative anaerobe with an optimum growth temperature of 37 °C, catalase and lysine decarboxylase positive, oxidase negative and capable of reducing nitrates to nitrites (Sussman, 1985). Isolates of *E. coli* have been classified by means of a serotyping scheme based on their somatic or cell wall (O), their capsular (K) and their flagella (H) antigens (Orskov et al., 1977; Sussman, 1985). Bacteriophage typing (Milch, 1978), and colicine typing (Gilies, 1978) have also been used for classification.

The non-pathogenic strains of *E. coli* have been widely used by biochemists and geneticists for elucidating the biosynthesis of macromolecules such as proteins and nucleic acids, the mechanisms of enzyme induction and repression, the role of nucleic acids in inheritance and many of the biochemical pathways that are known today. Recently, the non-pathogenic strains of *E. coli* have become pliant tools in the hands of genetic engineers for laboratory production of biomolecules of chemical, clinical and physiological importance. In parallel with these beneficial uses of *E. coli*, is the fact that some serotypes of *E. coli* have evolved over the years to become multipotent pathogens capable of causing a wide range of diseases of man and animals particularly in children and young animals (Tzipori et al., 1982; Newsome and Coney, 1985). The ubiquity and amazing range of adaptations of *E. coli* has made it the bacterium that is most frequently encountered by man. It can safely be stated that *E. coli* has established itself as man's unavoidable microbial companion.

**I.1 Health Problems Arising from *E. coli*:** Over the past few years, more than 70,000 cases of Gram-negative sepsis were reported in humans per year in the United States with one quarter (Wichterman et al., 1980) to one third (Wolff and Bennett, 1974) of the cases resulting in death. Two serotypes of *E. coli, O55:B5 and O111:B4* have been frequently implicated in many outbreaks resulting from enteropathogenic *E. coli* (Sussman, 1985). Human diseases attributed to *E. coli* are classified according to the major organs or systems affected by the bacterium or it's toxin(s). These are: (1) bacteremic diseases mainly affecting newborn and elderly or immunocompromised patients; (2) urinary tract infections affecting all age groups, (3) gastroenteritis associated with enteropathogenic serotypes, enteroinvasive strains and heat-stable or heat-labile toxic factors called enterotoxins, and (4) diseases characterised by vascular injuries (Karmali et al., 1985; Sherman et al., 1987). Enterotoxins are usually protein in nature and are specifically toxic to the cells of the intestinal mucosa. *Escherichia coli* is the most common isolate recovered from the cerebrospinal fluid and is responsible for about 50% of meningitis cases in newborn infants (Bortolussi et al., 1978). *Escherichia coli* has also been implicated in neonatal septicemia without meningitis and in pyelonephritis in children (Kaijser, 1973). There has also been some documentation on *E. coli* related agricultural problems. The most prominent problems include diarrheal diseases in calves (Sihvonen and Miettinen, 1985) and in neonatal piglets mainly during the first two weeks of life ( Moon et al., 1986), agalactia in pigs resulting from *E. coli*-induced mastitis (Hogg, 1988) and avian vasculitis (Rosenberger et al., 1985). *Escherichia coli* accounts for about 35% of neonatal diarrhea cases in piglets, 26% in calves, and 17% in lambs (Morris and Sojka, 1985).

### I.2 The Role of Endotoxin in the Pathogenesis of Gram-negative Sepsis:

Lysates of Gram-negative bacteria are known to exhibit toxicity and to initiate a number of pathophysiological conditions such as fever, hypotension, and disseminated intravascular coagulation (Westphal et al., 1986). The term endotoxin is used to describe the toxic factor because it is present within the bacterial cell and is released following cell lysis. Endotoxins are immunoreactive surface antigens found on the cell surface of Gram-negative bacteria, hence the term O-antigen is also used to describe endotoxins (Brade et al., 1988). In intact bacterial cells, they play an important role in the interaction of the bacteria with the host. Recognition of the endotoxins on the surface of invading bacteria triggers the host defense system to produce antibodies against the endotoxins. In addition to the endotoxins present on intact bacterial cells, endotoxins released following the breakdown of infecting bacterial cells make a significant contribution to the symptoms of bacteremia and sepsis. Endotoxins from these two sources have been implicated as the major contributing factor to the pathogenesis of Gram-negative infections or sepsis (Ziegler et al., 1982; Morrison and Duncan, 1985). Antibodies to common antigenic determinants on different endotoxins have been found to provide significant protection against Gram-negative bacteremia and sepsis (Greisman et al., 1973; Abdelnoor et al., 1981; Ziegler et al., 1982; Lindberg et al., 1983; Warren et al., 1987). Morrison and Duncan (1985) reported that endotoxins released from macrophages following *in vitro* phagocytosis of Gram-negative bacteria were equal to purified endotoxins in several endotoxic activities such as lethal toxicity, pyrogenicity, leucopenia, macrophage activation, B-lymphocyte mitogenicity, and induction of endotoxin cross-tolerance (Brade et al., 1988). These findings clearly suggest that administration of purified bacterial endotoxin is a useful model for studying some effects of bacterial infections. The advantage of this model over the administration of live bacteria is that the confounding effects of the metabolic activities of the bacteria are absent. Moreover, the dose of the endotoxin administered can be accurately quantified. In addition, endotoxin from bacteria that are killed during isolation procedures may account for part of the toxic symptoms that are initiated following the injection of an isolated bacterial culture.

I.3 **General Biochemistry of Endotoxin**: Endotoxins or O-antigens, are cell wall lipopolysaccharides found on the outer surface of Gram-negative bacteria (Sussman, 1985; Warren et al., 1987; Raetz, 1990). The lipopolysaccharide is complexed with the phospholipids of the outer membrane. Biologically active lipopolysaccharides may be prepared by hot phenol extraction (Rudbach et al., 1976), or cold aqueous butanol extraction (Morrison and Leive, 1975) of Gram-negative bacteria or by extraction of Gram-negative bacteria with a mixture of phenol, chloroform and petroleum ether (Galanos et al., 1969). The lipopolysaccharide molecule consists of three parts (Fig. I-1): the lipid A which is responsible for the direct toxicity of endotoxins, the core oligosaccharide and the outermost part called the O-specific chain, or O-antigen, which is responsible for the O-serogroup identity (Orskov et al., 1977; Dinarello and Wolff, 1982; Sussman, 1985).

I.3.1 **Lipid A**: The lipid A is structurally complex, consisting of a hydrophillic biphosphorylated glucosamine disaccharide heavily esterified with long chain fatty acids, this region of the molecule being hydrophobic (Brade et al., 1988). The lipid A isolated from *E. coli* and other enteric Gram-negative bacteria is asymmetrically acylated (Imoto et al., 1985; Kusumoto et al., 1985) in the sense that four fatty acids are associated with the nonreducing end of the disaccharide, while only two are linked to the reducing end glucosamine (Qureshi and Takayama, 1982). In *E. coli* it is a ß,1-6 linked disaccharide of glucosamine acylated with 3-

hydroxymyristate at positions 2, 3, 2′ and 3′ and phosphorylated at positions 1 and 4′. The two 3-hydroxy-acyl groups of the nonreducing glucosamine are further esterified with laurate and myristate (Raetz, 1990). On the other hand, lipid A from non-enteric organisms with the exception of *Hemophilus influenzae* (Helander et al., 1988) are symmetrical with respect to acyl chain replacement (Takayama et al., 1986).

Lipid A is linked to the unique eight carbon sugar, 2-keto-3-deoxyoctonic acid (KDO) of the core oligosaccharide. The 2-keto-3-deoxyoctonic acid (KDO) is also called 3-deoxy-D-manno-octulosonic acid (Brade et al., 1988). The KDO linkage of lipid A to the core oligosaccharide is acid labile. Free lipid A can therefore be obtained from LPS by boiling in aqueous 0.1 M HCl for 15 min (Raetz, 1990) or by boiling in 1% acetic acid for 2 h (Hancock and Reeves, 1976).

**I.3.2 The Core Oligosaccharide**: The core region of the lipopolysaccharide consists of a hetero-oligosaccharide that may be subdivided into the inner core domain that is proximal to the lipid A and the outer core domain that is distal to the lipid A. The inner core in most species of Gram-negative bacteria has a second unique seven carbon sugar called glycero-D-manno-heptose in addition to KDO (Raetz, 1990). The heptose moiety appears to play an essential role in the maintenance of outer membrane structure. Mutants that lack heptose are temperature insensitive (Boman and Monner, 1975; Hancock and Reeves, 1976), but unable to grow in the presence of bile acids and are hypersensitive to hydrophobic drugs (Raetz, 1990). The inner core is polyanionic because charged groups such as phosphoryl (Chaby and Szabo, 1975), pyrophosphoryl, phosphorylethanolamine or pyrophosphorylethanolamine (Brade et al., 1988) are often attached to the sugars. The outer core consists of hexoses, predominantly glucose, galactose, and N-acetylglucosamine (Jansson et al., 1981). Apart from performing the structural role of providing an attachment site for the O-antigen (O-specific side chain), the outer core appears not to have any established function.

**I.3.3 O-antigen or O-specific Side Chain**: The O-antigen is a heteropolysaccharide with repeating oligosaccharide units which bear the O-antigenic determinants (Brade et al., 1988), hence the O-antigen is also referred to as the O-specific polysaccharide. Repeating units ranging from trisaccharide to hexasaccharide units have been reported in Gram-negative bacteria (Parolis and Parolis, 1989; Raetz, 1990). Most O-antigen sugars are simple hexoses but some 6-deoxy- or 3,6-dideoxy-hexoses have been reported. The nature, sequence, type of linkage, and the type of substitution of the individual monosaccharide residues within a repeating unit are characteristic and unique for a given lipopolysaccharide and the parental bacterial strain. The O-specific chain is therefore species-specific and is involved in O-serogroup identity.

**I.4 Intestinal Endotoxin Pool and Endotoxemia**: The endotoxicity of lipopolysaccharide is expressed after its release within the body following the breakdown of infecting bacterial cells. Many of the endotoxic effects can be produced by parenteral administration of purified lipopolysaccharide. In clinical and experimental infections, endotoxin may be released from intact bacteria through natural death, antibiotic mediated bacteriolysis or by the action of the host defense system on the bacteria. In addition, a considerable amount of endotoxins may be ingested in food and water. Under different pathophysiological conditions, the gastrointestinal endotoxin pool could become sufficiently large to make endotoxemia of gut origin without bacteremia become clinically significant (Nolan, 1988). Pathophysiological conditions that could lead to endotoxemia of gut origin without bacteremia include; increased endotoxin production, increased absorption, or impaired detoxification of the toxin in the liver. When the gut barrier status is normal, very little endotoxin is absorbed (Ravin et al., 1960). However, experimental infection of rabbits (Cantey and Blake, 1977; Peeters et al., 1984; Batt et al., 1987), calves (Hall et al., 1985) and gnotobiotic pigs (Tzipori et al., 1985) with enteropathogenic *E. coli* has revealed brush border damage induced by the infection. Brush border damage has severe implications on intestinal transport and general homeostasis. Brush border damage could alter the balance in activities of enzymes such as the disaccharidases and the peptidases that are involved in the final stages of digestion and absorption of carbohydrates and proteins, respectively, since these enzymes reside within the brush border membrane. It is not clear whether endotoxin per se, can cause damage in the absence of any direct attack by bacteria. There is, therefore, a need to investigate the effect of endotoxin on the microscopic architecture of the gut and the possible effect on gut function.

I.5 **Biochemical Sequelae of Endotoxemia**: It has been believed for many years that endotoxin is responsible for the physiological and biochemical alterations associated with human Gram-negative bacterial shock (Wichterman et al., 1980), even though some measure of variation exists between the clinical pictures resulting from Gram-negative sepsis and endotoxic shock (Waisbren, 1964). Endotoxins have been implicated in the production of disease symptoms ranging from fever to potentially fatal endotoxic shock or septicemia (Wichterman et al., 1980; Sussman, 1985). Many of the toxic actions of endotoxin are mediated via tumour necrosis factor which is synthesized by the mononuclear phagocytes in response to endotoxin (Greve et al., 1990). Both natural and experimental severe infections are commonly accompanied by fever, trace mineral redistribution, increased net skeletal muscle catabolism, acute phase protein response characterised by an increase in the rate of protein synthesis in the liver (Sobrado et al., 1983), negative nitrogen balance (Long et al., 1977; Garlick et al., 1980a) and in some cases increased energy expenditure (Jepson et al., 1987).

Parenteral administration of bacterial endotoxins has produced changes ranging from fever (Hayashi et al., 1985), transient initial hyperglycemia followed by profound and terminal hypoglycemia (Wichterman et al., 1980), macrophage activation (Rylander and Beijer, 1987), elevation of acute phase protein concentrations, and a dose-dependent fall in plasma concentrations of zinc (Poole et al., 1984, Poole et al., 1986) as well as depression of plasma concentrations of a number of amino acids (Southorn and Thompson, 1986) in laboratory animals. In the guinea pig, the transitory increased rate of hepatic protein synthesis leading to an increase in the concentration of acute phase proteins occurred within 4 h following administration of endotoxin (Sobrado et al., 1983). After 4 h, the rate of hepatic protein synthesis in the endotoxin treated guinea pigs was not significantly different from the controls.

A review by van Miert and Frens (1968) documented signs of anorexia in the cat, dog, pig, goat, sheep, cow and horse during the febrile response to intravenous injection of bacterial endotoxin. Reduced gastric motility and decreased gastric secretions both in quantity and concentration of hydrochloric acid have also been reported in the rat (Brodie and Kundrats, 1964) and in the dog (Blickenstaff and Grossman, 1950). van Miert and Frens, (1968) using *E. coli*, 0111 $B_4$ lipopolysaccharide, reported rumen hypomotility in the cow, sheep and goat at a treatment dose of 0.05 μg/kg body weight and above, vomiting in the dog (1 μg/kg), swine (5 μg/kg) and cat (10 μg/kg) as well as diarrhea in the goat and dog (1 μg/kg) and in the mouse (30 μg/kg).

In healthy animals with intact skin, entry of most xenobiotics, including endotoxins, into the circulation is regulated by the gastrointestinal tract. Therefore, the effect of endotoxin on the gastrointestinal tract may play the most critical role in determining the course of events that culminate in endotoxic shock. For example, an increase in gut permeability would lead to greater influx of endotoxin into the circulation. If the circulating concentrations exceed the liver threshold capacity for detoxification, endotoxic shock results.

**I.5.1 Effect of Endotoxin on Absorption and Transport of Nutrients:** Experiments with rabbit ileum have provided morphological and biochemical evidence of damage to the microvillus membrane by enteropathogenic *E. coli* (Batt et al., 1987). In these experiments, loops of rabbit ileum were filled *in vivo* with enteropathogenic *E. coli* culture or saline and incubated for 45 min. At the end of the incubation period, the loops were washed with saline, mucosal biopsies were taken and cultured for up to 48 h. A marked increase in the release of brush border enzymes (zinc-resistant α-glucosidase or maltase and aminopeptidase N) was reported within 24 h postinfection while it took about 48 h for ultrastructural damage to become visible. Structural damage to the microvillus membrane could alter the intestinal barrier regulating xenobiotic entry into the circulation and distort carrier mediated transport of nutrients. The release of brush border enzymes due to damage to the brush border membrane could interfere with brush border digestion. Brush border digestion is important for the absorption of sugars and amino acids because a substantial amount of these compounds are absorbed from the intestinal lumen as disaccharides and dipeptides, respectively (Alpers, 1987). They are then hydrolysed to their constituent monosaccharides or amino acids, within the brush border membrane.

Enterotoxins released by enterotoxigenic *E. coli* are known to cause increases in intracellular cyclic AMP concentrations in both crypt and villus cells (Donowitz and Welsh, 1987) and/or increases in intestinal mucosal cyclic GMP concentrations (Field et al.,1978; Hughes et al., 1978; Guerrant et al., 1980). In the opossum, an increase in intracellular cyclic GMP concentrations in response to *E. coli* enterotoxin is not limited to the small intestine but includes the kidneys and testes where receptors for enterotoxin have also been found (Forte et al., 1989). Cyclic AMP and the $Ca^{2+}$-calmodulin complex have been implicated as second messengers responsible for regulating toxin-induced alterations in intestinal transport. It is not known whether endotoxins have effects that are similar to those produced by enterotoxins in this regard. Equally, the basis for metabolic changes such as hypoglycemia and depressed plasma levels of some amino acids and zinc associated with infection or endotoxin administration have not been fully explained. Since macromolecular nutrients are of no functional value to the animal until they are digested and absorbed, impairment of digestion and/or absorption are possible ways through which infection or endotoxemia could lead to some of these changes. However, very little is known about the effect of systemic *E. coli* infections or the influence of purified *E. coli* endotoxin on the functional integrity of the gastrointestinal tract, particularly as it relates to digestion of foods and absorption of nutrients from the gut.

Even though some authors (Beisel et al., 1967; Rosenberg et al., 1977) have speculated about the influence of infections on the absorptive capacity of the gut, only a few of these speculations are supported with direct experimental evidence. The picture of the state of the functional integrity of the gut's absorptive surface during infection and during the post-infection recovery period is far from being well understood. Although positive association between malabsorption of some nutrients with bacterial enteritis resulting from *Salmonella* (Gianella et al., 1971), *Shigella* (Rosenberg et al., 1977) and *Vibrio cholera* (Lindenbaum, 1965) have been reported, the type of nutrients affected and the nature of the effects are all dependent on the type, duration and the degree of severity of the infection. However, gastrointestinal involvement that has been reported in some animal species treated with bacterial endotoxin seems to suggest that systemic infection may have tremendous direct or indirect effects on the gastrointestinal tract. For example, Southorn and Thompson, (1986) reported increased salivation and anorexia in sheep injected intravenously with *E. coli* endotoxin. It is therefore apparent that the presence of bacteria or their toxins in the circulation could lead to disruption of the functional integrity of the gut (Grady and Keusch, 1971a and 1971b).

Two types of epithelial-toxin responses leading to malabsorption are thought to exist. The first type are pharmacotoxic agents which produce abnormalities in the function of the intestinal epithelium without producing morphological changes. An example of a pharmacotoxin is the cholera toxin (Field, 1971). The second type are cytotoxins which cause damage to the lining of the epithelium with or without invasion of the enterocytes. An example is the Shigella toxin (Grady and Keusch, 1971a and 1971b). While the effects of the pharmacotoxins seem to be readily reversible during recovery, the effects of the cytotoxins may persist for some time after apparent recovery from the infection. Damage to the mucosa of the intestine can cause disruption of digestive juice secretion both in quantity and quality as well as disruption of uptake and transport of nutrients by the jejunum. Although some information is available on enterotoxin induced-damage, it is not known whether endotoxin can induce similar damages.

### I.5.2 Enterocyte Energy Metabolism:

Digestion and absorption of food are the primary functions of the small intestine. Even though the small intestine is metabolically very active, studies relating to intermediary metabolism in the small intestine have not been given the prominence that those of the liver have received. The limiting factors responsible for this research emphasis include the preoccupation by researchers with its absorptive, transport and secretory functions, and the diverse population of cells found in this organ (Windmueller and Spaeth, 1978). Of the cell types present in the small intestine, the enterocytes are the most active metabolically and are directly involved in the final stages of carbohydrate and protein digestion (hydrolysis of disaccharides and dipeptides in the brush border membrane), and the absorption of the resulting endproducts. Therefore, endotoxin-induced alterations of the metabolism and the functions of the enterocytes may have dramatic impact on the overall performance of the small intestine. Moreover, the enterocytes can be readily isolated. This is the rationale behind studying the effects of endotoxin on metabolism of the enterocyte rather than the entire small intestine.

The enterocytes depend mainly on glucose, glutamine and ketone bodies for their energy supply under normal physiological conditions (Kimura, 1987), but glutamine is thought to be the principal fuel (Windmueller, 1982; Souba et al., 1985a; Kimura, 1987; Watford et al., 1987; Klimberg et al., 1990a). The amount of glutamine utilized is concentration dependent (Hanson and Parsons, 1977). Windmueller and Spaeth (1978) found that less than 1% of the fatty acid flux through the small intestine was oxidized. Most of the fatty acids were converted to triacylglycerols, and subsequently aggregated in chylomicra before they are released into the lymphatic circulation. *In vitro* vascular perfusion experiments in rats have revealed that fatty acids of chylomicra do not serve as respiratory fuels for the small intestine because of the absence of lipoprotein lipase, but some oleate is oxidized when complexed to albumin (Hulsmann et al., 1981). Long chain fatty acids are therefore not a major fuel for the small intestine under normal physiological conditions. Fuel utilization varies from one part of the intestine to another. Enterocytes isolated from the jejunum have a preference for glutamine while the colonocytes have a preference for butyrate (Fleming et al., 1991). Even within the same region of the intestine, fuel utilization varies with location of the enterocytes. For example, the rate of oxidation of acetoacetate, glycerol-1-phosphate and succinate in rat villi enterocyte mitochondria was found to be 12, 4 and 2 times the rate found in the crypt enterocyte mitochondria for these respective substrates (Hulsmann et al., 1970). The differential rates of substrate oxidation is possibly related to differential activities of key enzymes involved in the oxidation of these substrates. Histochemical examination revealed a virtual absence of succinate dehydrogenase activity and lower glycerol-1-phosphate dehydrogenase activity in the crypt than in the villi (Hulsmann et al., 1970).

**I.5.2.1 Effect of Endotoxin on Glucose Metabolism:** Altered carbohydrate metabolism leading to altered glucose homeostasis has been reported consistently during infection, sepsis or endotoxic shock (Long, 1977; Merrill and Spitzer, 1978; Kelleher et al., 1982; Lang et al., 1984; Lang and Spitzer, 1987). Progressive hypoglycemia resulting from endotoxic shock or live bacterial infection has been reported in rats, dogs and primates (Hinshaw, 1976). Circulatory factors, altered glucose production, abnormal glucose uptake or utilization and insulin or insulin-like influences have been implicated in accounting for the hypoglycemia (Hinshaw, 1976). The contribution of the liver to this overall picture of glucose metabolism during endotoxemia or sepsis has been studied extensively (McCallum and Berry, 1972; McCallum and Berry, 1973; McCallum, 1981; Deaciuc and Spitzer, 1986; Meszaros et al., 1991). Experiments with mouse liver, have revealed decreases in the incorporation of $^{14}$C-label from alanine, pyruvate or glucose into liver glycogen and in the activity of glycogen synthase (McCallum and Berry, 1973) as well as decreases in the activities of hepatic fructose-1,6-diphosphatase and glucose-6-phosphatase (McCallum and Berry, 1972). These observations are indicative of decreased glucose synthesis. In addition, defective oxidative metabolism in liver mitochondria has been reported during endotoxic shock in the rat (Mela et al., 1971). On the other hand, the contribution of the small intestine to the over all picture of defective glucose metabolism has not received equal attention even though the intestine is at a greater risk of exposure to endotoxin.

**I.5.2.2 Glutamine Metabolism in Enterocytes During Stress:** Although amino acids are primarily used as precursors for protein synthesis, they are also used as an energy source. It has been estimated that in man on an average diet, approximately 100 g of amino acids undergo oxidative degradation daily (Kovacevic et al., 1991).

In contrast to cells of most tissues in the body, enterocytes derive a major portion of their energy requirements from the oxidation of glutamine which incidentally is the most abundant free amino acid in the body (Souba et al., 1985b). When L-[U-$^{14}$C]glutamine was administered intraluminally in rats that had been fasted overnight, only 34% of the glutamine was recovered unchanged in venous blood collected from jejunal segments (Windmueller and Spaeth, 1975; Windmueller, 1980). Of the portion metabolised, 60% of the $^{14}$C carbon was recovered in $CO_2$, with the remaining $^{14}$C appearing in amino acids such as proline, citrulline, alanine, ornithine and glutamate and in organic acids mostly lactate. Stressful conditions such as injury, surgery (Souba and Wilmore, 1983), infection (Wilmore et al., 1988) or glucocorticoid administration (Souba et al., 1985c) give rise to increased glutamine demands by the intestine. In the postabsorptive state, gut glutamine demand is met by glutamine export from the muscle. Thus, an increase in gut glutamine demand imposes a drain on the muscle free glutamine pool. Dramatic declines in free glutamine concentrations in skeletal muscle and/or in the circulatory system have been reported during infection (Askanazi et al., 1980; Kapadia et al., 1985; Milewski et al., 1982), injury (Aulick and Wilmore, 1979; Albina et al., 1986; Caldwell, 1989), surgery (Vinnars et al., 1975; Kapadia et al., 1985), burns (Ardawi, 1988) and other forms of trauma. Radiation injury to the intestinal mucosa is often associated with destruction of proliferative crypt cells, decreased villi height and ulceration or necrosis of the gut epithelium (Klimberg et al., 1990a, Berthrong, 1986). These effects are ameliorated by prophylactic feeding of glutamine-enriched elemental diets (Klimberg et al., 1990b).

Glutamine homeostasis is threatened during critical illnesses because the net rate of glutamine utilization exceeds the net rate of glutamine production (Souba et al., 1990). The skeletal muscle is a major site of glutamine synthesis in the body (Meister, 1980) and is therefore the most important source of glutamine in postabsorptive states (Felig, 1975); Darmaun et al., 1986). During stress-induced increases in the demand for glutamine in the body (see Fig. I-2), the amount synthesized in the body might fall short of what is required. This would call for an increase in the supply of glutamine from exogenous sources.

I.5.2.3 **Effect of Glutamine Supplementation on Gut Permeability During Stress:** There is substantial evidence that tight junctions play a major role in regulating epithelial permeability by influencing paracellular flow of fluids and solutes. Tissues that have regional permeability differences such as the nephrons also have regional variations in tight junction structure that correlate inversely with resistance (Pricam et al. 1974). Stress-induced disruption of tight junctions of toad urinary bladder mucosa is accompanied with penetration by macromolecules that are normally excluded (Wade et al., 1973).

Supplementation of parenteral formulae with glutamine has been associated with preservation of normal jejunal architecture in the rat (Barber et al., 1990), decreased bacterial translocation and improved survival during experimental enterocolitis (Fox et al., 1988). Bacterial translocation was determined in this study by culturing blood samples to detect bacteremia. Oral glutamine administration accelerates the healing of the small intestine following radiation injury (Klimberg et al., 1990a). It would appear that dietary glutamine supplementation could be employed to maintain glutamine homeostasis during stresses such as endotoxemia or sepsis, and consequently prevent or ameliorate the adverse developments highlighted in Fig I-2.

**I.6 Effect of Infection or Endotoxemia on Protein Turnover:** Protein turnover may be defined as the continual synthesis, degradation and resynthesis of protein in the body. When the rate of synthesis is greater than the rate of degradation there is net accumulation of protein in the body and the organism is said to be in positive nitrogen balance. On the other hand, if the rate of degradation is higher than the rate of synthesis there is net loss of tissue protein and the animal is then said to be in negative nitrogen balance. The body's responses to infection are generally catabolic, characterised by growth suppression, negative nitrogen balance, and in some cases increased energy expenditure (Jepson et al., 1987). Negative nitrogen balance has been reported in subjects with experimentally induced tularemia (Beisel, 1966) and in septic rats (Powanda et al., 1972). Several studies (Keilman, 1977; Biesel, 1984; Rennie, 1985) suggest that catabolic losses of skeletal muscle protein nitrogen account for a substantial component of the negative nitrogen balance. The rate of protein degradation is significantly increased in rats infected with *E. coli* (Fagan and Goldberg, 1985) leading to muscle wasting (Choo et al., 1989).

*Escherichia coli* injection in chicks by Klasing and Austic, (1984a) resulted in decreased rates of protein synthesis in the gastrocnemius and increased rates of synthesis in the liver, bursa, spleen and thymus. These changes were accompanied with increased aggregation of polysomes in the bursa, spleen and thymus and increased aggregation of free, but not bound, polysomes in the liver. In a similar experiment, Klasing and Austic, (1984b) revealed increased rates of protein degradation in muscle, spleen and thymus but there were no changes in the rate of degradation in the liver and bursa in response to *E. coli* infection. It should be pointed out here that the rate of protein degradation in the liver was estimated by measuring arginine release. Even though arginine is not metabolized to urea by chick liver, contributions from *de novo* synthesis of arginine are a likely source of error. Baracos et al. (1983) reported enhancement of protein degradation in muscle by endogenous pyrogen. Induction of prostaglandin synthesis was implicated as the mechanism through which endogenous pyrogen acts since indomethacin, an inhibitor of prostaglandin synthesis, was found to abolish the enhanced proteolysis.

Experimentally induced endotoxemia in rats by subcutaneous injection of endotoxin from *E. coli, serotype 0127:B8* resulted in a 60-100% increase in muscle protein degradation and a 52% decrease in protein synthesis (Jepson et al., 1986). Most of the studies aimed at unravelling the mechanism of infection-induced nitrogen loss have been carried out on the skeletal musculature since it is the largest reservoir of relatively labile protein in the body. In view of the fact that the rate of protein turnover in the intestinal mucosa is high relative to that reported for other tissues in the body, the contribution of the intestinal mucosa to nitrogen loss during infection is worth exploring. Moreover, the intestine is more at risk in coming in contact with bacteria or their toxins than any other internal organ of the body.

A number of methods are available for the determination of protein synthesis. Most of these methods are based on the incorporation of an isotopically labelled essential amino acid that is not extensively metabolised. The most commonly used methods in this category include the constant infusion method based on the earlier work of Waterlow and Stephen (1967) and the flooding dose technique (Garlick et al., 1980b).

On the other hand, there are not many reliable methods for accurate measurement of protein degradation. Determination based on tyrosine release in the presence of cycloheximide has been used to estimate protein degradation in skeletal muscle. The method is based on the fact that tyrosine is not metabolised to any appreciable extent by skeletal muscle

(Klasing and Austic, 1984b) and that cycloheximide prevents re-incorporation of tyrosine into protein. The tyrosine release method is easy to use but is only reliable if the tissue involved does not metabolise tyrosine. Another method that is now in use for measuring protein degradation *in vitro* is the measurement of the release of a radiolabelled amino acid from a prelabelled protein (Sugden and Fuller, 1991). It is not known whether tyrosine release can be used to reliably measure protein degradation in the small intestine.

I.7 **Hypothesis and Objectives**: The overall hypothesis tested in this thesis is that endotoxin affects small intestinal structure, transport functions, and amino acid and protein metabolism.

Therefore the objectives of the thesis were to determine the effect of endotoxin on -

1 the transport and metabolism of glucose by the small intestine,
2 the transport and metabolism of amino acids by the small intestine,
3 enterocyte glutamine metabolism,
4 protein degradation by the enterocytes,
5 intestinal morphology, enterocyte turnover, and disaccharidase activity

and to determine the effect of dietary glutamine supplementation on -

(1) glutamine homeostasis in the body,
(2) endotoxic response by the small intestine.

Fig. I-1. Cell wall of Escherichia coli showing the location and a generalised structure of the lipopolysaccharide. The repeating unit consists of 3 - 6 monosaccharide residues, and n is variable. KDO represents one or more 2-keto-3-deoxyoctonic acid or 3-deoxy-D-manno-octulosonic acid residue(s). Adapted from Raetz, (1990).

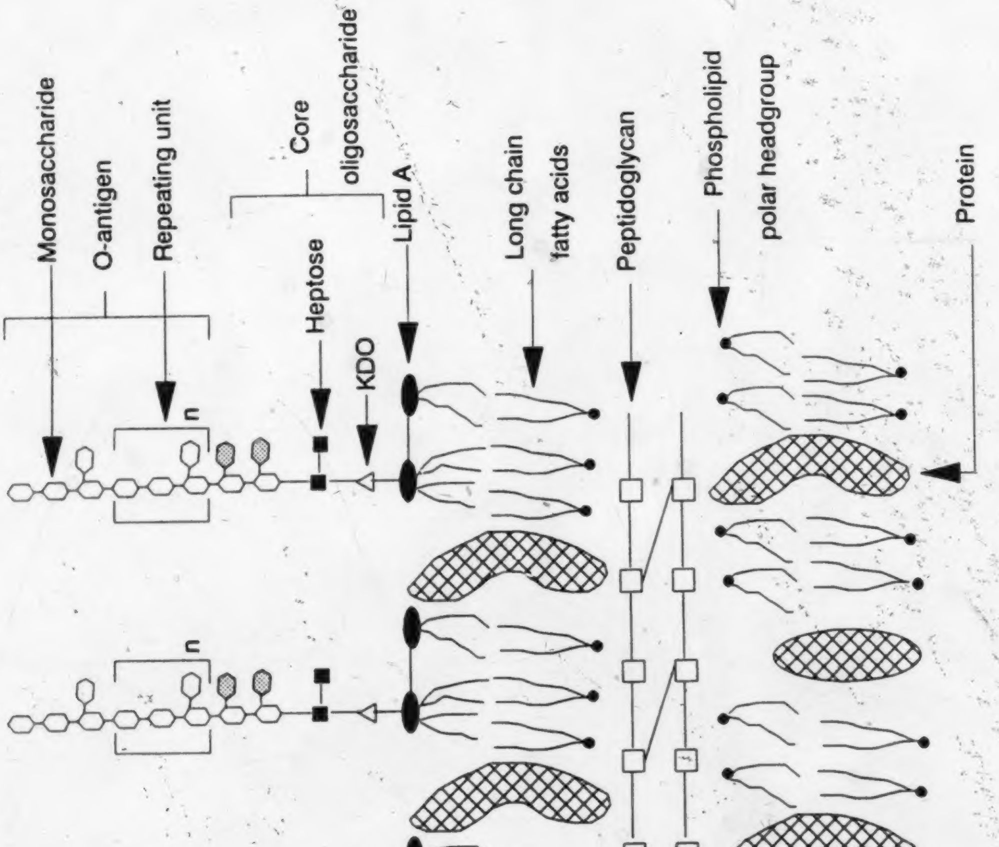
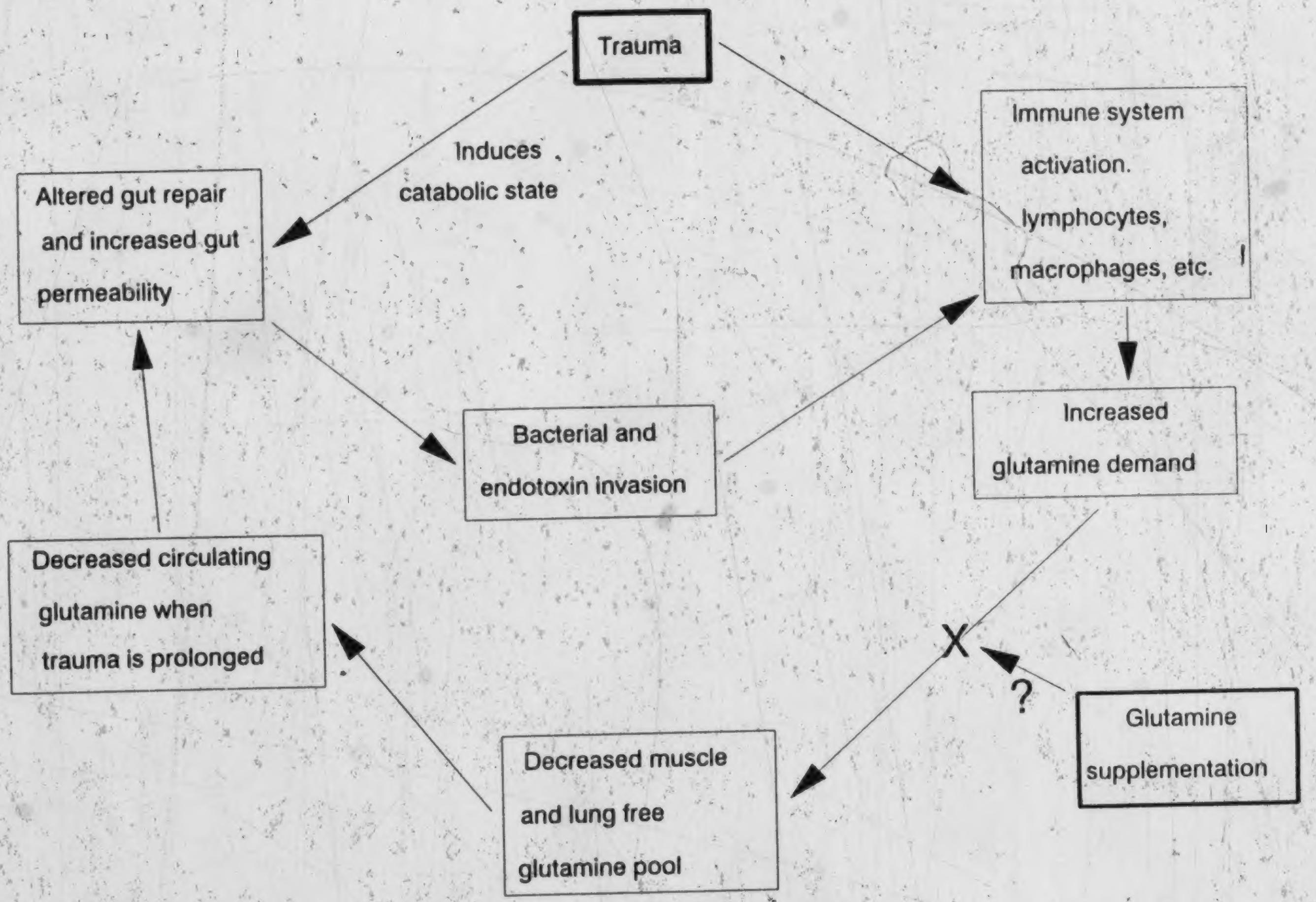

Lipopolysaccharide
(Endotoxin)
Outer
membrane
lipid bilayer
Periplasm
Inner
membrane
lipid bilayer
Monosaccharide
O-antigen
Repeating unit
n
Core
oligosaccharide
Heptose
KDO
Lipid A
Long chain
fatty acids
Peptidoglycan
Phospholipid
polar headgroup
Protein

Fig I-2. Involvement of glutamine in the body's response to trauma and a possible role for dietary glutamine supplementation.


Trauma
Induces catabolic state
Altered gut repair and increased gut permeability
Immune system activation. lymphocytes, macrophages, etc.
Bacterial and endotoxin invasion
Increased glutamine demand
Decreased circulating glutamine when trauma is prolonged
X
?
Glutamine supplementation
Decreased muscle and lung free glutamine pool

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# II. Effect of Endotoxin on Glucose and Amino Acid Transport, and Metabolism of Glucose in the Rat Small Intestine.

## Introduction:

*Escherichia coli* serotypes 055:B5 and 0111:B4 have been implicated in a number of gastro-enteritis outbreaks in humans (Sussman, 1985) and in animals (Morris and Sojka, 1985) and have therefore attracted considerable attention. Altered glucose metabolism (Kelleher et al., 1982) and plasma amino acid concentrations (Southorn and Thompson, 1986) following sepsis or injection of purified bacterial endotoxin have been reported in experimental animals including rats and sheep. Continuous infusion or bolus injection of *E. coli* endotoxin is commonly associated with hypoglycemia (Lang et al., 1984), decreased plasma concentrations of a number of amino acids (Southorn and Thompson, 1986) and decreased gastrointestinal tract motility (Lohius et al., 1988). Endotoxin injection at concentrations ranging from 10-100 ng per gram body weight has been reported to give biological responses in various species (van Miert and Frens, 1968).

The metabolic events leading to altered glucose and amino acid dynamics following bacterial endotoxin administration have not been well documented. In particular, very little is known about alterations in intermediary metabolism in the small intestine during the presence of endotoxins. Changes in metabolic activity and functional integrity, particularly transport function, of the small intestine may be a significant part of the mechanism through which changes in plasma concentrations of several metabolites of dietary origin is achieved.

Various methods are available for the measurement of nutrient transport across the small intestine. Csaky (1984) gave an exhaustive review of the commonly used methods. Some of these include: (1) measurement of substrate disappearance from the lumen of a loop of small intestine that is intact with it's blood supply, (2) disappearance of a substrate between two cannulated points, (3) appearance of a substrate in intestinal loop (everted loop technique) or disappearance of a substrate from the loop (uneverted loop technique), (4) the use of intestinal sheets in Ussing chambers and (5) the use of isolated epithelial membrane vesicles. In this study, the effects of *E. coli (O55:B5)* endotoxin on metabolism and apparent transport of glucose and amino acids by the rat ileum were investigated in Ussing chambers using two approaches involving: (1) measurement of short circuit current ($I_{sc}$) across the intestinal sheet and (2) direct quantification of the appearance of substrate $^{14}$C radioactivity in the serosal compartment.

## Materials and Methods:

Male Sprague Dawley rats were obtained one week prior to starting the experiment and were provided rat chow and water *ad libitum*. The rats were exposed to a 12 h light/dark cycle and were cared for in accordance with the guidelines of the Canadian Council on Animal Care. At the time of the experiment the rats weighed between 225 and 280 g. [U-$^{14}$C]glucose was obtained from New England Nuclear, Mississauga, ON., Canada, while $NaH^{14}CO_3$ was obtained from ICN Radiochemicals, Montreal, PQ., Canada. Endotoxin-free saline was obtained from Travenol Canada Inc., Mississauga ON., Canada. Lipopolysaccharide (endotoxin) from *E. coli (O55:B5)* was obtained from Sigma Chemical Co., St. Louis MO., U.S.A.

**Determination of the Amino Acid Composition of the Ileal Fluid**: In order to determine the amino acid composition of postabsorption ileal lumen fluid, 10 male Sprague Dawley rats, previously fed rat chow *ad libitum*, were fasted for 12 h before killing by decapitation. The ileum was immediately removed and its contents were forced into separate tubes for each rat by a stream of air from a syringe. The tubes were then centrifuged at 3000 x g and the clear fluid saved for analysis of free amino acids by HPLC (Jones and Gilligan, 1983). The amino acid concentrations in the incubation medium subsequently used for the amino acid transport experiments were formulated to equal those measured in the ileal lumen fluid thereby providing experimental conditions that were as close as possible to physiological state free amino acid concentrations.

**Tissue Preparation and Mounting**: Each rat was fasted for 12 h before anaesthesia with 55 mg/kg of sodium pentobarbital administered intraperitoneally. The abdomen was quickly opened and a short segment of the proximal ileum was rapidly removed and the heart was cut open to kill the rat. The lumen of the ileal segment was rinsed with a stream of ice cold Krebs-Ringer buffer (KRB, see Appendix 1) from a syringe to remove food debris. The segment was then opened along the mesenteric border according to the procedure of Thomson (1983) and rinsed in cold KRB that had been saturated with carbogen (95% $O_2$:5% $CO_2$). Several small pieces of the intestine 1.5 cm in length were cut from the segment into fresh KRB constantly gassed with carbogen and maintained cold in an ice bath. The pieces of tissue were then mounted on Lucite Ussing chambers with an internal radius of 0.5 cm (Ussing and Zerahn, 1951). The smooth muscle layer was carefully removed and the chambers were quickly fitted to the reservoir containing KRB maintained at 37 °C and constantly gassed with carbogen (see Appendix 6 for the diagrammatic representation of an Ussing chamber). The *E. coli* endotoxin solution in endotoxin-free saline was added as described below under each specific treatment to give a final concentration of 20 ng endotoxin /ml of medium.

**Measurement of Glucose Transport-Dependent Changes in Electrical Properties of Ileal Tissue Slices:** When an intestinal tissue slice mounted in a KRB-filled Ussing chamber is short circuited so that both sides of the slice are exposed to the same potential, an $I_{sc}$ flows through the tissue as a result of the active movement of positive ions from the mucosal to the serosal compartment (Armstrong and Garcia-Diaz, 1984). This $I_{sc}$ can be measured with a microameter and its magnitude following the addition of 3-O-methylglucose is proportional to the transport of this solute (Misfeldt and Sanders, 1985). The effect of endotoxin on the electrical properties of ileal tissue slices was measured at 4 concentrations of 3-O-methylglucose. Ileal tissue slices from each rat were mounted as described above to 1 control and 1 treatment chamber for each of the 4 3-O-methylglucose concentrations and then connected to the reservoir containing 9.8 ml of KRB in each compartment. One hundred microliters of saline or endotoxin solution (2 μg/ml) were added to the mucosal and serosal compartments of each of the control and treatment chambers, respectively. The agar bridges of the Ussing chambers were connected and a preincubation period of 20 min was allowed for equilibration during which the residual voltage and the $I_{sc}$ were determined every 5 min by means of a millivolt meter and a microameter, respectively. Any tissue showing a residual voltage of less than 1 mV was discarded on account of poor viability.

At the end of the 20 min preincubation period, 100 μl of 0.05 M, 0.25 M, 0.5 M or 1.0 M 3-O-methylglucose and an equivalent volume of saline were added to the mucosal and serosal compartments of the chambers, respectively. The final concentrations of 3-O-methylglucose in the 4 treatment and control chambers were therefore 0.5 mM, 2.5 mM, 5.0 mM or 10 mM, respectively. The voltage and the $I_{sc}$ across the two compartments of each chamber separated by the intestinal slice were measured at 2 min intervals for 10 min. Then 100 μl of saline containing 0.5 mM phlorizin were added and measurements were made for an additional 10 min at 2 min intervals. Phlorizin inhibits sodium-dependent glucose transport (Cheung and Hammerman, 1988). Correction for fluid resistance (Tai and Tai, 1981) was made on the values measured.

**Transport and Metabolism of Glucose and Amino Acids:** After a 20 min preincubation period, the mucosal compartment of the chambers was adjusted to contain 0.15, 1.0, 2.5, 5.0, or 6.0 mM [U-$^{14}$C]glucose and incubated for 1 h. During incubation, $^{14}CO_2$ produced from [U-$^{14}$C]glucose was trapped in liquid scintillation cocktail containing 5.0 g PPO, 0.2 g POPOP, 200 ml ethanolamine, 300 ml methyl cellosolve and 500 ml toluene At the end of the incubation period, $CO_2$ dissolved in the incubation medium was released by acidification with 0.5 ml 50% perchloric acid and collected in the trapping solution. The perchloric acid also terminated the metabolic activities of the tissue. The efficiency of the $^{14}CO_2$ trapping system was 91.94% as determined with $NaH^{14}CO_3$. Radioactivity was measured in a Beckman, LS 5801 liquid scintillation counter.

The specific radioactivity of the starting [U-$^{14}$C]glucose solution was measured at the beginning of the experiment. This specific radioactivity was then converted to dpm per μmole glucose carbon by dividing the dpm per μmole [U-$^{14}$C]glucose by 6. To determine background radioactivity, the tissue was mounted as usual but the 100 μl $^{14}$C-glucose solution was replaced with saline.

For measurement of apparent amino acid transport, amino acid concentrations found in the ileal lumen fluid were provided in the mucosal compartment incubation medium after a preincubation period of 20 min. At the end of 30 min incubation period, solutions in the two compartments were collected, the volumes were measured and analysed for amino acid composition by HPLC. For background determinations, the tissue was mounted as usual but no amino acids were added to the chamber compartments. The amount of each amino acid appearing in the serosal compartment represents the background amino acid transport in the absence of exogenous amino acids. All apparent amino acid transport values were corrected for background values.

**Statistical Analysis:** Differences between the control and the endotoxin-treated animals were analysed by Student's t-test.

## Results:

**Effect of Endotoxin on Glucose Transport-Dependent Short Circuit Current:** Endotoxin did not affect the glucose transport dependent $I_{sc}$ across the ileal tissue preparation in the presence of 0.5 mM 3-O-methylglucose but it decreased $I_{sc}$ at 3-O-methylglucose concentrations ranging from 2.5 - 10 mM (Fig. II-1). The Michaelis constant ($K_M$) was not affected by the endotoxin treatment while $I_{sc(max)}$ was reduced from 47.6 to 34.5 $\mu$A. The addition of 0.5 mM phlorizin 10 min after the addition of 5 mM 3-O-methylglucose reduced $I_{sc}$ in both the control and endotoxin treated tissues but did not significantly alter the difference between the control $I_{sc}$ and the $I_{sc}$ of the endotoxin treated tissue (Fig. II-2).

**Effect of Endotoxin on Apparent Transport and Metabolism of Glucose:** Tables II-1 and II-2 show the results of the studies on glucose transport and metabolism, respectively, in the presence and absence of the endotoxin. Bacterial endotoxin had no effect on transport and metabolism of glucose at low glucose concentrations but at 5 mM glucose there was a significant ($P<0.05$) decrease in apparent glucose transport in the presence of endotoxin. At glucose concentrations ranging from 2.5 mM to 6.0 mM, there was an increase in the proportion of glucose taken up that was metabolised to carbon dioxide in the presence of endotoxin. However, there was no significant increase in the total amount of glucose oxidised to carbon dioxide. Almost all the glucose taken up at the very low concentration of 0.15 mM was oxidised $CO_2$.

**Amino acid Composition of Ileal Lumen Fluid:** The amino acid found in the highest concentration in the ileal lumen fluid was leucine followed by alanine and glutamic acid while tryptophan concentration was the lowest (Table II-3). Taurine, an amino acid not present in the rat diet, was found in the ileal lumen fluid at a concentration of 5 mM.

**Effect of Endotoxin on Apparent Transport of Amino Acids:** In the presence of endotoxin the apparent transport of four amino acids was reduced ($P<0.05$). Aspartic acid transport was reduced from 142 ± 28 to 68 ± 19, glutamic acid from 253 ± 36 to 146 ± 47, serine from 173 ± 26 to 101 ± 37 and threonine from 200 ± 22 to 137 ± 40, nmol $cm^{-2}$ $h^{-1}$, representing a decrease of 51.7%, 42.2%, 42.0 and 31.7%, respectively

(Table II-4). Citrulline, an amino acid not present in the amino acid solution added to the mucosal compartment of the chamber was released into the serosal compartment at a rate of 22 and 28 nmol $cm^{-2}$ $h^{-1}$ in the absence and presence of endotoxin, respectively. Background values for tissue release determined in incubations in the absence of exogenous amino acids were not more than 8 % of values found in the presence of amino acids.

**Discussion:**

There was good agreement concerning the effect of endotoxin on glucose transport in the two experimental approaches used in this study. The magnitude of the $I_{sc}$ is proportional to the transport of 3-O-methylglucose, a glucose analogue that is transported like glucose but is not metabolised (Misfeldt and Sanders, 1985). The decrease in $I_{sc}$ observed following endotoxin treatment at 3-O-methylglucose concentrations ranging from 2.5 to 10 mM suggests that bacterial endotoxin inhibits glucose transport. This type of inhibition was not suppressed by increasing substrate concentration since $I_{sc(max)}$ was decreased from 47.6 to 34.5 μA in the presence of endotoxin. The shape of the $I_{sc}$ versus [S] plot (Fig. II-1) suggests that the transport component affected by endotoxin is carrier-mediated since $I_{sc}$ appeared to show signs of approaching a maximum at 10 mM 3-O-methylglucose. The addition of phlorizin, an inhibitor of $Na^+$-dependent glucose transport (Misfeldt and Sanders, 1985, Cheung and Hammerman, 1988), 10 min after 3-O-methylglucose addition reduced the $I_{sc}$ in both control and endotoxin treated tissue but did not alter the relative difference between the two (Fig. II-2). It was expected that if the effect of endotoxin is on an active transport component, the addition of phlorizin would make the control tissue $I_{sc}$ approach the values measured for the endotoxin treated tissue $I_{sc}$. The present results therefore suggest that the effect of endotoxin on the active transport component may be negligible.

The glucose transport values reported in Table II-1 are reported as glucose carbon transport because some of the glucose taken up from the mucosal compartment would have been metabolised via glycolytic or TCA cycle reactions to various intermediates which in turn may have been released into the serosal compartment along with [U-$^{14}$C]glucose itself. No attempt was made to partition the radioactivity measured in the serosal compartment into glucose and it's metabolites other than $CO_2$. The fact that relatively different proportions of glucose taken up by the tissues could have been metabolized may explain why the effect of endotoxin on glucose carbon transport measured with 2.5 mM [$^{14}$C]glucose was not statistically significant whereas the change in $I_{sc}$ measured with 2.5 mM 3-O-methylglucose was significantly ($P<0.05$) lower when measured in the presence of endotoxin. The finding that almost all the glucose transported at the very low concentration of 0.15 mM was oxidised (Table II-2) suggests that even though glutamine is the preferred fuel of the intestine (Watford et al. 1987), a certain amount of glucose is required for providing energy for transport and other energy-requiring processes taking place within the cell especially when the amount of glutamine available is low.

In the present study, it was found that endotoxin treated intestine metabolised a higher proportion of glucose taken up for transport than the control intestine. Nevertheless, the total quantity of glucose metabolised to carbon dioxide did not change significantly ($P>0.05$) with endotoxin treatment.

Since taurine is not a dietary constituent, the ileal lumen fluid taurine likely arose from taurocholic acid produced in the liver. Citrulline was found in the medium after incubation but was not detected in the ileal lumen fluid. It is likely that in the live animal, citrulline synthesized in the small intestine ( Windmueller and Spaeth, 1981) is exported to the kidney for arginine biosynthesis (Dhanakoti et al., 1990).

The four amino acids whose apparent transport was reduced ($P<0.05$) by endotoxin are either dicarboxylic (aspartic and glutamic acid) or hydroxylated (serine and threonine). It should be pointed out that the ileal lumen fluid amino acid concentrations employed in this study may not necessarily be in excess of the $K_M$ for transport of all the amino acids studied. A possibility exists that the amino acids affected may be sharing the same carrier or have different carriers that respond in a similar manner to endotoxin. In the present study, the most drastic effect of endotoxin was on aspartic acid. The endotoxin may therefore be considered to be acting at the level of the carriers of these particular amino acids. Shrands (1973) has proposed membrane involvement of endotoxin in mediating it's biological effects. Reduced amino acid ($^{3}$H-$\alpha$-aminoisobutyric acid) transport in skeletal muscle in response to endotoxemia induced by *E. coli, O111:B4* endotoxin has been reported in rats by Warner et al. (1989).

The reduction of intestinal transport of these amino acids is not likely to have direct serious nutritional consequences because these amino acids are not essential. However, glutamic acid is required for glutamine synthesis. In view of the apparent importance of glutamine during trauma and/or infections, restricted glutamic acid transport could limit the availability of glutamic acid for glutamine synthesis and may pose problems in two ways: (1) The immunocytes such as lymphocytes and macrophages metabolize a relatively large amount of glutamine and therefore the immune system may be performing suboptimally when glutamine availability becomes limited; (2) Glutamic acid is the amino group donor in the biosynthesis of many non essential amino acids in the body through transamination reactions (Lehninger, 1982), therefore a limitation in glutamic acid availability could reduce transamination reactions.

**Fig. II-1: Effect of Endotoxin on 3-O-methylglucose Transport.** Endotoxin was present at a concentration of 20 ng/ml in the incubation medium. Each point represents a mean of 6 determinations + SEM. *Different ($P<0.05$) from control. The $K_M$ of 5.2 mM was virtually unaffected by the endotoxin treatment while $I_{sc(max)}$ was decreased from 47.6 to 34.5 μA.

Fig. II-1: Effect of Endotoxin on 3-O-Methylglucose Transport

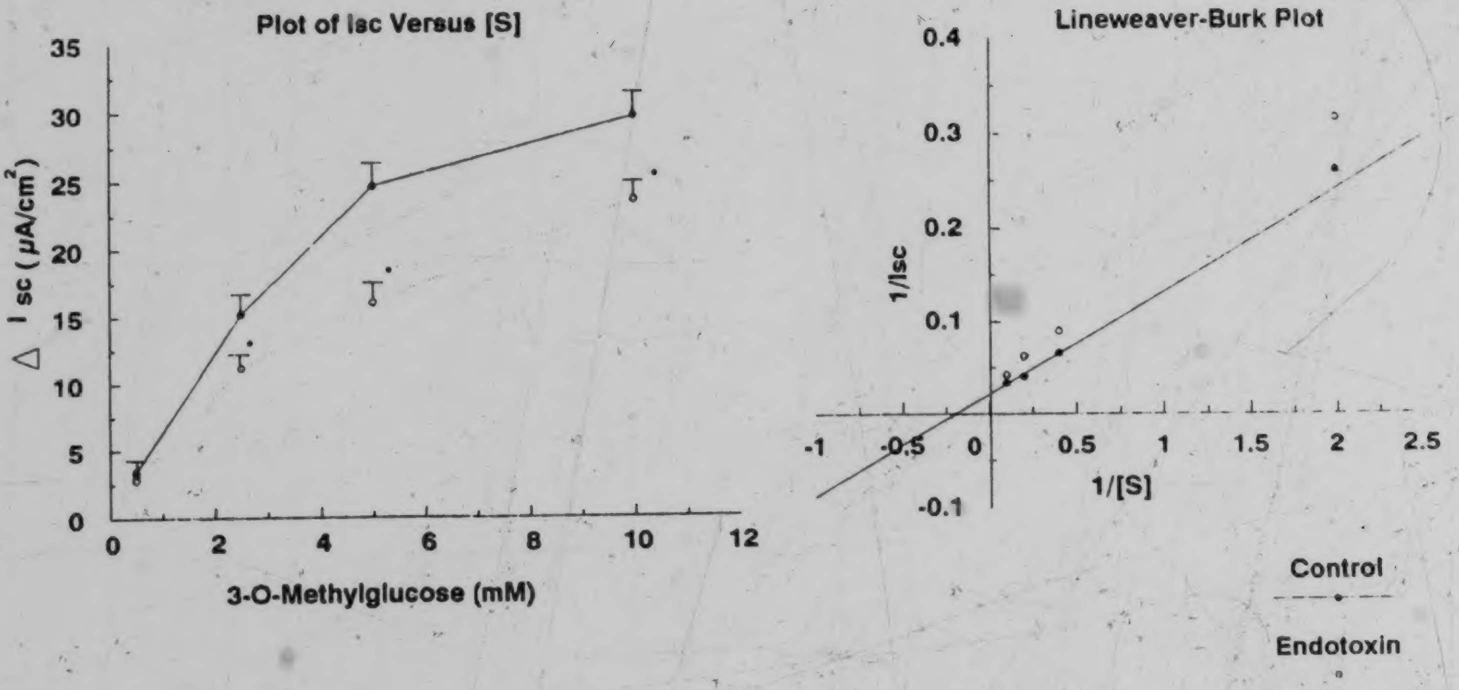

**Fig. II-2: Effect of Endotoxin on the Glucose Transport-Dependent Short Circuit Current of Ileal Tissue.**

Endotoxin was present at a concentration of 20 ng/ml in the incubation medium. After a 20 min preincubation period 5 mM 3-O-methylglucose was added to the incubation medium. Phlorizin (0.5 mM) was added 10 min later. Each point represents a mean of 6 determinations + SEM.

* Different ($P<0.05$) from control.

Fig. II-2: Effect of Endotoxin on the Glucose Transport-Dependent Short Circuit Current of Ileal Tissue

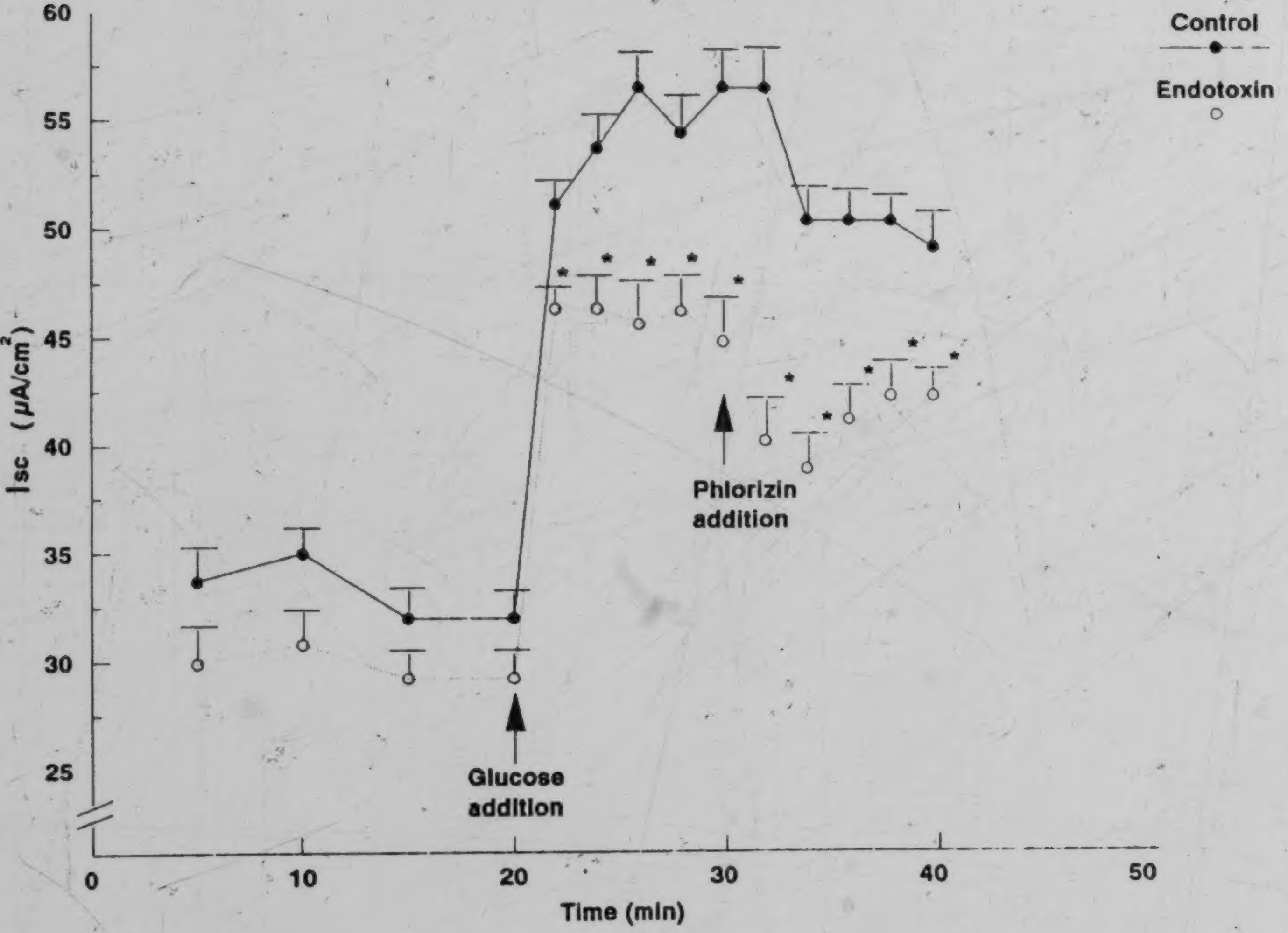

Table II-1. Transport of $^{14}$C-Glucose Carbon Across Flat Sheets of Rat Ileum In Vitro in the Presence or Absence of *E. coli* Endotoxin

| Glucose | Transport of $^{14}$C-Glucose Carbon | |
|---|---|---|
| | Control | Endotoxin |
| mM | $\mu$mol cm$^{-2}$ h$^{-1}$ | mmol cm$^{-2}$ h$^{-1}$ |
| 0.15 | 0.012 ± 0.001 | 0.011 ± 0.002 |
| 1.00 | 0.920 ± 0.130 | 0.930 ± 0.150 |
| 2.50 | 1.979 ± 0.170 | 1.720 ± 0.140 |
| 5.00 | 3.501 ± 0.131 | 3.100 ± 0.120* |
| 6.00 | 3.810 ± 0.230 | 3.047 ± 0.170* |

Transport of glucose carbon was calculated as $^{14}$C-glucose carbon transported across the ileal tissue to the serosal side. *Different from control ($P<0.05$). $n=6$

Table II-2. Oxidation of Glucose Carbon to $CO_2$ by Flat Sheets of Rat Ileum *In Vitro* in the Presence or Absence of *E. coli* Endotoxin

| Glucose | Glucose carbon metabolised to carbon dioxide | | % Transported glucose carbon oxidised to carbon dioxide[1] | |
|---|---|---|---|---|
| | Control | Endotoxin | Control | Endotoxin |
| mM | $\mu mol\ cm^{-2}\ h^{-1}$ | | % | |
| 0.15 | 0.055 ± 0.002 | 0.050 ± 0.002 | 82.20 ± 1.82 | 80.47 ± 3.40 |
| 1.00 | 0.123 ± 0.003 | 0.128 ± 0.003 | 11.81 ± 0.66 | 12.12 ± 0.80 |
| 2.50 | 0.116 ± 0.004 | 0.127 ± 0.003 | 5.03 ± 0.23 | 6.07 ± 0.34* |
| 5.00 | 0.169 ± 0.004 | 0.175 ± 0.004 | 4.60 ± 0.24 | 5.34 ± 0.25* |
| 6.00 | 0.160 ± 0.010 | 0.164 ± 0.011 | 4.02 ± 0.35 | 5.10 ± 0.54* |

[1] $\%CO_2 = ({}^{14}CO_{2\,collected} / {}^{14}C$ activity in medium $+ {}^{14}CO_{2\,collected}) \times 100$

* Different from control ($P<0.05$). n=6

Table II-3: Amino Acid Composition of Rat Ileal Lumen Fluid After 12 Hours Fasting

| Amino acid | Concentration mM |
|---|---|
| Alanine | 11.5 ± 2.1 |
| Arginine | 8.7 ± 2.3 |
| Asparagine | 4.1 ± 0.6 |
| Aspartic acid | 5.6 ± 0.8 |
| Glutamic acid | 11.1 ± 1.4 |
| Glutamine | 5.4 ± 1.2 |
| Glycine | 7.5 ± 0.8 |
| Histidine | 2.3 ± 0.4 |
| Isoleucine | 6.6 ± 1.3 |
| Leucine | 13.2 ± 3.0 |
| Lysine | 9.0 ± 1.8 |
| Methionine | 3.5 ± 0.9 |
| Phenylalanine | 6.0 ± 1.3 |
| Serine | 8.2 ± 1.2 |
| Taurine | 5.0 ± 1.9 |
| Threonine | 6.8 ± 1.0 |
| Tryptophan | 2.1 ± 0.3 |
| Tyrosine | 5.1 ± 1.2 |
| Valine | 7.7 ± 1.5 |

n=10

**Table II-4**: The Effect of *E. coli* Endotoxin on Apparent Amino Acid Transport by Rat Intestine In Vitro

| **Amino acid** | Control<br>nmol cm$^{-2}$ h$^{-1}$ | Endotoxin-treated<br>nmol cm$^{-2}$ h$^{-1}$ |
|---|---|---|
| Alanine | 608 ± 48 | 692 ± 129 |
| Arginine | 292 ± 26 | 310 ± 61 |
| Asparagine | 56 ± 8 | 50 ± 10 |
| Aspartic acid | 142 ± 28 | 68 ± 19* |
| Citrulline | 22 ± 4 | 28 ± 4 |
| Glutamic acid | 253 ± 36 | 146 ± 47* |
| Glutamine | 32 ± 15 | 20 ± 14 |
| Glycine | 343 ± 26 | 332 ± 56 |
| Histidine | 47 ± 5 | 42 ± 7 |
| Isoleucine | 301 ± 34 | 336 ± 66 |
| Leucine | 513 ± 63 | 566 ± 106 |
| Lysine | 333 ± 34 | 373 ± 66 |
| Methionine | 148 ± 19 | 167 ± 29 |
| Phenylalanine | 220 ± 25 | 206 ± 56 |
| Serine | 173 ± 26 | 101 ± 37* |
| Taurine | 128 ± 23 | 154 ± 23 |
| Threonine | 200 ± 22 | 137 ± 40* |
| Tryptophan | 69 ± 20 | 66 ± 13 |
| Tyrosine | 164 ± 20 | 156 ± 38 |
| Valine | 308 ± 35 | 345 ± 58 |

* Statistically different from control values ($P<0.05$), n-7.

a Amino acid not added to the mucosa compartment

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# III Effect of *Escherichia coli* Endotoxin on Glutamine Metabolism and Protein Degradation in Isolated Enterocytes[1]

## Introduction:

Altered glucose metabolism (Kelleher et al., 1982) and plasma amino acid concentrations (Southorn and Thompson, 1986) following sepsis or injection of purified bacterial endotoxin have been reported in experimental animals including the rat and sheep. Continuous infusion or bolus injection of *E. coli* endotoxin is commonly associated with hypoglycemia (Lang et al., 1984), decreased plasma concentrations of a number of amino acids (Southorn and Thompson 1986), decreased gastrointestinal tract motility (van Miert and Frens, 1968; Lohuis et al., 1988). Endotoxin injection at concentrations ranging from 10-100 ng per gram body weight has been reported to give biological responses in various species (van Miert and Frens, 1968).

Under normal physiological conditions, a significant amount of glutamine is used by the small intestine for meeting its energy requirements (Watford et al., 1987; King et al., 1983). In the post-absorptive state or during fasting, enterocyte glutamine metabolism may play an important role in the provision of carbon skeletons in the form of alanine for hepatic gluconeogenesis. Therefore, during the hypoglycemic condition associated with endotoxemia, alterations in enterocyte alanine production may contribute to the establishment of hypoglycemia. Very little is known about the metabolism of the small intestine in the presence of bacterial endotoxins. It is not known whether net enterocyte alanine production is decreased or increased in the presence of endotoxin. Therefore one objective of this study was to determine the effects of endotoxin on net alanine production by enterocytes in the presence of glutamine, and/or glucose as energy substrates. Since glutamine is metabolized to alanine through glutamate, net glutamate production was also examined.

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[1] A version of this chapter has been published. Ameh, D.A. and Thompson, J.R. (1990) In Amino Acids: Chemistry, Biology and Medicine. Lubec, G and Rosenthal, G.A. Eds. ESCOM Science Pub., B.V. pp. 1009-1016.

Marked changes in protein metabolism including an increase in whole body protein turnover rate have been reported following sepsis or trauma (Tomkins et al., 1983) and vaccination (Garlick et al., 1980a). Subcutaneous administration of E. coli endotoxin to the rat has been reported to cause a 60-100% increase in the rate of skeletal muscle protein degradation (Jepson et al., 1986). The effect of endotoxin on enterocyte protein degradation has not been reported.

The methods available for the measurement of protein degradation only provide estimates of the process. Determinations based on tyrosine release in the presence of cycloheximide and in the absence of tyrosine in the incubation medium have been used to estimate protein degradation in skeletal muscle in which tyrosine is not metabolised to any appreciable extent (Tischler et al., 1982). The tyrosine release method is relatively easy to use but it is not known if it can be used to reliably estimate protein degradation in enterocytes. Determination of [$^3$H]phenylalanine release from prelabelled tissue proteins is another method for measuring protein degradation (Wu and Thompson, 1990). Therefore, the second objective of this study was to determine the effect of endotoxin on enterocyte protein degradation and to determine protein degradation by measuring [$^3$H]phenylalanine release from prelabelled enterocyte protein. The possibility of using tyrosine release to estimate protein degradation in isolated enterocytes was also explored.

Materials and methods:

**Materials**: Male Sprague Dawley rats weighing 250-310 g provided food and water ad libitum were used in all experiments unless stated otherwise. The animals were exposed to a 12 h light/dark cycle and were cared for in accordance with the guidelines of the Canadian Council on Animal Care. Endotoxin (from *E. coli (055:B5))* was obtained from Sigma Chemical Co., St. Louis MO., U.S.A. L-[2,6-$^3$H]phenylalanine was obtained from Amersham International, Oakville, ON., Canada. Endotoxin-free saline was obtained from Travenol Canada Inc., Mississauga ON., Canada.

**Isolation of Rat Enterocytes**: The method of Reiser and Christiansen (1971) as modified by Watford et al. (1979) was used for isolating enterocytes from the small intestine. Following a 12 h fast each rat was killed and the abdomen was rapidly opened. A 90 cm segment of the small intestine beginning 5 cm below the pylorus was excised and cut into two equal portions. The intestinal contents were flushed out of each portion with ice-cold Krebs-Ringer bicarbonate buffer (KRB) saturated with carbogen ($O_2$:$CO_2$ (19:1)). A further 20 ml of KRB saturated with carbogen were passed through the intestine to remove mucus. One end of each portion was ligated with 3-0 Dexon and the intestine was filled with KRB containing 1.5 mg/ml hyaluronidase and 2.5 mg/ml bovine serum albumin. The other ends were ligated and the portions were incubated in 100 ml of KRB for 15 min at 37 °C with constant agitation and gassing with carbogen. The contents were then discarded and the intestines refilled with pre-gassed KRB. Following ligation, the intestinal portions were gently patted for 1 min with finger tips on ice-blocks covered with a polythene sheet to release enterocytes into the intestinal lumen. The KRB containing enterocytes from both portions of the intestine was pooled in polythene tubes and centrifuged at 1000 x g for 3 min. The cells were washed 3 times with cold KRB and resuspended in 10 ml KRB for subsequent metabolic studies. Viability of the isolated enterocytes was determined by trypan blue exclusion and lactate dehydrogenase leakage. A detailed description of the method of assessment of enterocyte viability is given in Appendix 3. The average cell density (dry weight of enterocytes per ml of suspension) was determined by the method of Kimmich (1970). Known volumes of enterocyte suspension and equal volumes of KRB were each dried to a constant weight in an oven maintained at 105 °C. The dry weight of cells was determined by subtracting the dry weight of KRB solute from that of the cell suspension.

**Measurement of Net Glutamate and Alanine Production and Tyrosine Release by Enterocytes**: Tyrosine release and the net production of glutamic acid and alanine were measured in enterocytes from each of six animals. Two hundred microlitres of the enterocyte preparation were added to 3.1 ml of incubation media containing KRB alone or KRB plus 10 mM glucose, 5 mM glutamine, or 5 mM glutamine plus 10 mM glucose in the presence or absence of *E. coli* endotoxin (50 ng/ml). The incubation medium was constantly gassed with carbogen throughout the incubation period. Two hundred μl of the enterocyte suspension placed in 3.3 ml 4% perchloric acid at time 0 min were used to measure the amount of free amino acids before incubation. At the end of the 1 h incubation period, the reaction was terminated by addition of 0.2 ml 70% perchloric acid bringing the total volume to 3.5 ml and the medium was centrifuged at 2500 x g for 10 min. The pellet was homogenised in 1.0 ml 4 % perchloric acid. One hundred microliters of the incubation media and 200 μl of the tissue homogenate were analysed for free amino acids by high performance liquid chromatography (HPLC) according to the method of Jones and Gilligan (1983). Net production of an amino acid equals total free amino acid in cells plus medium after the 1 h incubation minus the total free amino acid present at time 0 min while net release equals total free amino acid present in the medium at the end of the incubation period. As a follow up on increased tyrosine release in the presence of 5 mM glutamine, observed in the first experiment, another experiment was conducted to determine the effect 5 mM glutamine on essential amino acid release in the presence and absence of plasma concentrations other amino acids.

**Measurement of Protein Degradation:** Eight young male Sprague Dawley rats weighing approximately 100 g were fed rat chow and water *ad libitum* until 12 h prior to killing. Twenty four hours prior to the end of the experiment, each rat was injected intraperitoneally with 0.50 ml of physiological saline containing 0.4 mM L-[2,6-$^3$H]phenylalanine (150 $\mu$Ci/100 g body weight). The rats were housed in individual metallic cages and all urine and faeces were collected to prevent radioactive contamination. The enterocytes were isolated as described earlier. The freshly harvested enterocytes were washed with five 10 ml portions of a 0.4 mM solution of non-radioactive phenylalanine in KRB to remove free [$^3$H]phenylalanine. To ascertain the effectiveness of removal of free [$^3$H]phenylalanine, the washes were counted for radioactivity. After five washes the radioactivity in the wash was similar to the background value. The cells were resuspended in 10 ml KRB and used for the protein degradation studies.

Sixteen 200 $\mu$l portions of the enterocyte suspension from each rat were incubated with one of the following treatments; 3.1 ml KRB containing 0.4 mM non-radioactive phenylalanine (blank) or 3.1 ml KRB containing 0.4 mM non-radioactive phenylalanine and 5 mM glutamine or 5 mM glutamine plus 10 mM glucose or 10 mM glucose for 1 h in the presence or absence of 50 ng/ml endotoxin. In addition, two incubation conditions were used for contrast, one in which amino acids were absent from the media and the other in which plasma concentrations of amino acids (Ardawi, 1988) were present. At the end of the 1 h incubation period, the reaction was terminated by addition of 0.2 ml of 70% perchloric acid. The enterocytes were spun down at 1000 x g for 5 min and the supernatant collected. This supernatant contained amino acids released into the incubation medium plus amino acids from the intracellular free amino acid pool. One ml of the supernatant was counted for radioactivity in a liquid scintillation spectrometer to estimate the amount of [$^3$H]phenylalanine released from protein.

To determine the amount of protein-bound [$^3$H]phenylalanine, 1 ml of the cell suspension was hydrolysed with 6 N HCl at 110 °C for 24 h. The hydrolysate was evaporated to dryness and taken up in 3 ml of water. The solution was analysed for phenylalanine by HPLC and 1 ml of the solution was counted for [$^3$H]phenylalanine radioactivity. The rate of protein degradation was calculated by dividing the [$^3$H]phenylalanine radioactivity released into the incubation medium during the 1 h incubation period by the specific radioactivity of protein-bound [$^3$H]phenylalanine. The fractional rate of degradation in per cent per day was calculated by multiplying the nmol phe released per h per mg by 24 times 100%, then divided by nmol phe per mg of enterocytes. The proportion of protein-bound $^3$H activity in enterocytes not due to phenylalanine was determined by paper chromatography.

Results were analysed using two way analysis of variance (Steel and Torrie, 1980). Differences between means were tested for significance using S.N.K. method. P values <0.05 were taken to be significant.

## Results and Discussion:

### Glutamine Metabolism and Amino Acid Production by the Enterocytes:

In the first study, the effects of endotoxin and the energy substrates glutamine and glucose on net alanine and glutamate production by rat enterocytes were studied. Substantially greater (P<0.001) amounts of glutamate and alanine were produced by enterocytes incubated in the presence of 5 mM glutamine or 5 mM glutamine plus 10 mM glucose than by enterocytes incubated in the presence of 10 mM glucose alone (Table III-1). These observations are consistent with the well established fate of glutamine in the small intestine (Watford et al., 1979; Hanson and Parsons, 1980). The finding that glutamine is extensively metabolised to glutamate is in agreement with the high glutaminase activity reported in the small intestine (Watford et al., 1987; Nagy and Kretchmer, 1986; Pinkus and Windmueller, 1977). Further metabolism of glutamate leads to the production of alanine. The inclusion of endotoxin in the incubation media did not change ($P>0.05$) net production of the glucogenic substrates, alanine and glutamate. In the presence of 5 mM glutamine, significantly ($P>0.05$) more alanine was produced than when enterocytes were incubated in 10 mM glucose. The inclusion of 10 mM glucose in the presence of 5 mM glutamine further increased ($P<0.05$) net alanine production by approximately 100%, suggesting that a portion of the carbon skeleton of alanine arose from glucose. Hanson and Parsons (1980) proposed that glutamate derived from glutamine leaves the mitochondria to be transaminated in the cytosol with extramitochondrial pyruvate derived from pyruvate produced from glucose. In addition to the shifts in alanine and glutamate production, there were increases in the net production of the other amino acids similar to those reported in Table III-2.

A relatively high concentration of taurine (10.54 ± 1.26 nmol/mg) was also found in the enterocytes. Taurine concentrations did not change during the 1 h incubation period regardless of the treatment, implying that this amino acid is not produced within the enterocytes. Quirk et al. (1989) also reported high concentrations of taurine in enterocytes isolated from the chicken jejunum. Enterocytes may serve as a taurine reserve which can be readily mobilised for hepatic biodetoxification of xenobiotics as tauroconjugates and may also be involved in the recycling of bile acids as tauroconjugates.

Relatively high concentrations of ethanolamine were also found in the incubation medium following the 1 h incubation period. The total amount of ethanolamine approximately doubled during the incubation period from 4.18 ± 1.13 to 10.15 ± 1.00 nmol/mg tissue. The exact source of ethanolamine is not clear. However, Quirk et al. (1989) have reported the presence of a serine ethanolamine phosphodiester in chicken enterocytes which may serve as the precursor of ethanolamine in this tissue. It must therefore be stressed that ethanolamine is unsuitable as an internal standard for quantitative determination of amino acids in enterocyte samples. The energy substrates, glucose and glutamine, had no effect on enterocyte ethanolamine concentrations during the period of incubation. The addition of E. coli endotoxin to the incubation media also did not influence ethanolamine concentration (data not shown).

**Tyrosine Release and Protein Turnover:**

The possibility of using tyrosine release as a measure of protein breakdown in rat enterocytes was explored, bearing in mind that the release of amino acids from a tissue depends upon the relative rates of protein synthesis and degradation as well as membrane amino acid transport and the extent of formation and metabolism of the particular amino acid within the tissue. Glutamine (5 mM) caused a significant increase in tyrosine release from the enterocytes which was not influenced by the addition of glucose (Table III-3). This is suggestive of an increased rate of protein breakdown following the addition of glutamine if tyrosine is not metabolised in the enterocytes. However, as can be seen from Table III-2, the three fold increase in net tyrosine release cannot be completely explained by an increased rate of proteolysis alone since the relative increase in net release of an essential amino acid such as lysine did not respond in a similar manner. Therefore it remains possible that enterocytes may produce tyrosine or that the presence of 5 mM glutamine influences amino acid efflux from the enterocytes into the medium. Hydroxylation of phenylalanine to tyrosine is one possibility but glutamine-induced hydroxylation of [$^3$H]phenylalanine by isolated enterocytes could not be demonstrated qualitatively *in vitro* (see Appendix 4).

To obtain a more direct estimation of protein breakdown, rats were injected with [$^3$H]phenylalanine 24 h before sacrifice to prelabel enterocyte proteins. The release of [$^3$H]phenylalanine from the prelabelled proteins was measured as an index of the relative rates of protein breakdown in control and endotoxin treated enterocytes and in enterocytes exposed to glutamine and glucose. The rate of [$^3$H]phenylalanine release provides a good estimate of protein degradation since the large amount of free phenylalanine in the extracellular free amino acid pool from the incubation medium is overwhelmingly larger than the intracellular pool thereby minimizing the chance of reincorporation of released [$^3$H]phenylalanine into enterocyte protein. Only 4% of the protein bound $^3$H radioactivity in enterocytes isolated from rats 24 h after [$^3$H]phenylalanine injection was not due to phenylalanine. This was determined by comparing the radioactivity in the phenylalanine band with the total radioactivity in paper chromatogram.

Inclusion of E. coli endotoxin in the incubation medium extensively increased ($P<0.001$) the rate of enterocyte protein degradation both in the presence and in the absence of plasma concentrations of amino acids (Table III-4). In contrast to the response of tyrosine to the addition of glutamine to the incubation medium, glutamine did not affect the rate of protein degradation. The degradation rates found in this study correspond to fractional degradation rates of 122%-142% per day in control and 328-450% in enterocytes from endotoxin treated rats. These results are in contrast to the lack of effect of endotoxin on tyrosine release (Table III-3). In other laboratories, subcutaneous administration of E. coli endotoxin to the rat has been reported to cause a 60-100% increase in the rate of muscle protein degradation, and a 52% fall in the rate of muscle protein synthesis (Jepson et al., 1986). The fractional rate of protein synthesis was increased by 35% in the liver in the same experiment. The fractional rates of protein degradation found in control enterocytes in this study are close to the value of 119% per day observed *in vivo* by others (Garlick et al., 1980b). The effect of endotoxin on the rate of protein synthesis in the small intestine was not measured in their experiment. The results of the present study demonstrate that enterocyte protein degradation is more sensitive to bacterial endotoxin treatment than skeletal muscle. The three fold increase in the rate of protein degradation observed in the present study was much higher than the 60-100% increase reported in skeletal muscle by Jepson et al. (1986).

Addition of 10 mM glucose, 5 mM glutamine or 5 mM glutamine plus 10 mM glucose to the incubation media did not influence the rate of protein degradation (Table III-4), but did increase the net release of tyrosine three fold (Table III-2). The measurement of tyrosine release for assessing protein breakdown therefore appears unsuitable in rat enterocytes. These preliminary findings demonstrate that glutamine may influence tyrosine metabolism or transport but has little or no effect on protein metabolism in enterocytes *in vitro*. Bacterial endotoxins appear to have a very significant effect on the rate of protein degradation in enterocytes. Considerable additional work is required to delineate the effects of bacterial endotoxins on amino acid and protein metabolism in these cells.

Table III-1: Effect of Endotoxin and Glutamine on Net Alanine and Glutamate Production by Rat Enterocytes

Enterocytes isolated from the small intestine of the rat were incubated for 1 h at 37 °C in the presence of 10 mM glucose or 5 mM glutamine or 5 mM glutamine plus 10 mM glucose . Means within a column followed by different superscripts are significantly different ($P<0.05$) from non endotoxin treated control values. n=6

| | Alanine (nmol/h/mg enterocyte) | | Glutamate (nmol/h/mg enterocyte) | |
|---|---|---|---|---|
| Substrates | Control | Endotoxin | Control | Endotoxin |
| 10 mM glucose | $14.2 \pm 0.4^{a}$ | $15.5 \pm 1.0^{a}$ | $5.4 \pm 0.2^{a}$ | $6.3 \pm 0.6^{a}$ |
| 5 mM gln | $25.3 \pm 2.4^{b}$ | $25.4 \pm 2.9^{b}$ | $126.9 \pm 11.4^{b}$ | $111.7 \pm 9.2^{b}$ |
| 5 mM gln & 10 mM glucose | $52.8 \pm 4.8^{c}$ | $50.5 \pm 0.9^{c}$ | $121.8 \pm 12.7^{b}$ | $106.0 \pm 12.6^{b}$ |

**Table III-2: Effect of Glutamine and Glucose on Net Tyrosine and Essential Amino Acid release by Rat Enterocytes in the Absence or Presence of Amino Acids**

Isolated enterocytes from rats were incubated for 1 h at 37 °C in KRB or KRB plus 5 mM glutamine or 5 mM glutamine plus 5 mM glucose in the presence or absence of plasma amino acid concentrations. Means within the same row followed by different superscripts are significantly different ($P<0.05$). n=8

| | Amino acid Net Release (nmol/h/mg tissue) | | |
|---|---|---|---|
| Amino Acid | In KRB | 5 mM Gln | 5 mM Gln & 10 mM glucose |
| Absence of amino acids | | | |
| Threonine | $5.26 \pm 0.89^{a}$ | $11.47 \pm 2.24^{b}$ | $10.53 \pm 1.72^{b}$ |
| Tryptophan | $1.08 \pm 0.21^{a}$ | $1.88 \pm 0.25^{b}$ | $1.92 \pm 0.22^{b}$ |
| Methionine | $1.09 \pm 0.33^{a}$ | $3.27 \pm 0.57^{b}$ | $2.82 \pm 0.36^{b}$ |
| Valine | $5.82 \pm 0.79^{a}$ | $10.25 \pm 2.18^{b}$ | $9.09 \pm 1.58^{b}$ |
| Phenylalanine | $2.79 \pm 0.37^{a}$ | $5.91 \pm 0.78^{b}$ | $5.43 \pm 0.50^{b}$ |
| Isoleucine | $3.82 \pm 0.44^{a}$ | $6.50 \pm 1.21^{b}$ | $5.46 \pm 0.84^{b}$ |
| Leucine | $6.88 \pm 1.28^{a}$ | $12.39 \pm 2.12^{b}$ | $11.02 \pm 1.36^{b}$ |
| Lysine | $12.38 \pm 2.14^{a}$ | $17.78 \pm 3.18^{a}$ | $15.91 \pm 2.18^{a}$ |
| Tyrosine | $3.93 \pm 0.41^{a}$ | $11.64 \pm 0.62^{b}$ | $11.49 \pm 0.65^{b}$ |
| Presence of amino acids | | | |
| Threonine | $21.57 \pm 3.61^{a}$ | $17.95 \pm 4.23^{a}$ | $15.63 \pm 1.98^{a}$ |
| Tryptophan | $4.70 \pm 0.48^{a}$ | $3.72 \pm 0.43^{b}$ | $2.75 \pm 0.48^{c}$ |
| Methionine | $4.63 \pm 0.52^{a}$ | $3.69 \pm 0.64^{b}$ | $1.76 \pm 0.79^{c}$ |
| Valine | $9.46 \pm 0.93^{a}$ | $7.15 \pm 1.31^{b}$ | $2.70 \pm 1.47^{c}$ |
| Phenylalanine | $4.15 \pm 0.64^{a}$ | $2.54 \pm 1.15^{b}$ | $0.92 \pm 1.36^{c}$ |
| Isoleucine | $3.99 \pm 0.26^{a}$ | $3.68 \pm 0.58^{a}$ | $2.51 \pm 0.46^{b}$ |
| Leucine | $10.37 \pm 0.77^{a}$ | $9.47 \pm 1.20^{a}$ | $6.87 \pm 1.36^{b}$ |
| Lysine | $20.27 \pm 4.03^{a}$ | $15.25 \pm 4.05^{a}$ | $14.27 \pm 3.50^{b}$ |
| Tyrosine | $3.84 \pm 0.40^{a}$ | $12.93 \pm 1.04^{b}$ | $12.89 \pm 0.81^{b}$ |

**Table III-3: Effect of E. coli Endotoxin on Tyrosine Release by Rat Enterocytes in the Absence of Amino Acids**

Enterocytes isolated from the small intestine of the rat were incubated for 1 h at 37 °C in Krebs-Ringer buffer or in the presence of 5 mM glutamine or 5 mM glutamine plus 10 mM glucose or 10 mM glucose. Means within a column followed by different superscripts are significantly different ($P<0.001$). n=6

| | Net tyrosine release | |
|---|---|---|
| | Control (nmol/mg enterocyte) | Endotoxin (nmol/mg enterocyte) |
| Krebs-Ringer buffer | $2.53 \pm 0.15^a$ | $2.86 \pm 0.12^a$ |
| 5 mM glutamine | $14.51 \pm 0.81^b$ | $14.70 \pm 0.84^b$ |
| 5 mM gln & 10 mM glucose | $14.88 \pm 1.07^b$ | $15.09 \pm 0.84^b$ |
| 10 mM glucose | $2.36 \pm 0.06^a$ | $2.68 \pm 0.14^a$ |

**Table III-4: Effect of E. coli Endotoxin and Different Energy Substrates on Protein Degradation by Rat Enterocytes in the Absence or Presence of Amino Acids**

Enterocytes isolated from the small intestine of rats containing [$^{3}$H]phenylalanine prelabelled protein, were incubated for 1 h at 37 °C in the presence or absence of plasma concentrations of amino acids plus 0.4 mM non-radioactive phenylalanine and 5 mM glutamine or 5 mM glutamine plus 10 mM glucose. Protein degradation was determined by measuring the release of [$^{3}$H]phenylalanine from the enterocytes. Means within the same row followed by different superscripts are significantly different (P<0.001). n=8

| | Protein Degradation (nmol Phe/h/mg tissue) | |
|---|---|---|
| | Control | Endotoxin |
| **Amino acids absent from incubation media** | | |
| Krebs-Ringer buffer | 1.72 ± 0.07$^{a}$ | 5.11 ± 0.38$^{b}$ |
| Plus 5 mM gln | 1.75 ± 0.23$^{a}$ | 6.38 ± 0.88$^{b}$ |
| Plus 5 mM gln & 10 mM glucose | 1.70 ± 0.19$^{a}$ | 4.38 ± 0.41$^{b}$ |
| Plus 10 mM glucose | 1.77 ± 0.22$^{a}$ | 5.03 ± 0.80$^{b}$ |
| **Amino acids present in incubation media** | | |
| Krebs-Ringer buffer | 1.67 ± 0.19$^{a}$ | 4.75 ± 0.42$^{b}$ |
| Plus 5 mM gln | 1.89 ± 0.25$^{a}$ | 5.14 ± 0.67$^{b}$ |
| Plus 5 mM gln & 10 mM glucose | 1.67 ± 0.21$^{a}$ | 4.90 ± 0.70$^{b}$ |
| Plus 10 mM glucose | 1.63 ± 0.18$^{a}$ | 5.19 ± 0.46$^{b}$ |

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# IV Effect of *Escherichia coli* Endotoxin on Rat Enterocyte [$^3$H]Thymidine Incorporation and Disaccharidase Activity

## Introduction:

The primary functions of the gastrointestinal tract include digestion and absorption of foods and protection of the body from a variety of noxious materials present in the tract. In order to perform these roles effectively, the gastrointestinal tract must provide it's full compliment of digestive enzymes as well as maintain it's structural integrity. The small intestine may be exposed to a number of systemic and luminal insults including antinutritional factors, injury, inflammation, infection or bacterial endotoxins. The integrity of the mucosal lining is essential to its role as a barrier to enteric bacteria, preventing host intoxication or invasion by microorganisms (Berg and Garlinton, 1979; Wilmore et al., 1988; Li et al., 1989). In normal healthy individuals, the epithelial cells prevent bacterial translocation through the paracellular route (Marin et al., 1983). It has been suggested that in critically ill patients the barrier function of the intestinal epithelium is severely compromised and gut permeability is increased thereby exposing the patient to intraluminal bacteria and their toxins (Hollander et al., 1986). However, the permeability study that led them to this suggestion was carried out with polyethylene glycol-400 and may not necessarily be identical with bacterial endotoxin or lipopolysaccharide which has a much higher molecular weight.

The manner in which the intestinal epithelium maintains its functional integrity has not been clearly documented but appears to be related to its ability to replace epithelial cells as fast as they are extruded from the villi tips. Although factors such as total starvation or protein malnutrition known to produce intestinal atrophy have been reported to cause a decrease in cell production and cell migration (Alpers and Kinzie, 1973), the mechanisms responsible for regulating the rate of enterocyte production at the crypts, migration along the villi to the extrusion zone and extrusion itself are not well understood. Leblond and Walker (1956) postulated the 'wound healing' hypothesis in which it was suggested that extrusion of cells from the villi tips leaves gaps which are subsequently filled by cells migrating from the crypt. For the structural and functional integrity of the villi to be maintained, the rate of production of cells must be closely matched to the rate of extrusion.

There is a paucity of information concerning the effect of bacterial endotoxins on enterocyte turnover. Since circulating concentrations of endotoxins are elevated during Gram negative infection or sepsis (Sturk and ten Cate, 1983), studies involving systemic administration of purified endotoxin can improve our understanding of Gram negative bacterial diseases. Therefore, one objective of this study was to examine the influence of systemic *E coli* endotoxin on [$^3$H]methylthymidine incorporation into enterocyte DNA. It should be pointed out that thymidine incorporation into DNA does not measure directly, the **cell cycle time** (the time interval between two successive divisions), the **growth fraction** (the fraction of cell population devoted to proliferation) or cell migration rate, and is consequently not a measure of enterocyte turnover. However, thymidine incorporation measurement provides information on changes in DNA synthetic rates and by inference, changes in the rate of enterocyte production. An increase in thymidine incorporation is indicative of increased cell production rate in the crypts.

Luminal digestion of dietary carbohydrate results in the production of disaccharides and oligosaccharides (Alpers, 1987). The disaccharides are further digested by disaccharidases while oligosaccharides are further hydrolysed by the action of α-limit dextrinase, and glucoamylase. Isomaltase which hydrolyses isomaltose is also active on α,1-6 links in α-dextrins. Disaccharidase activity is quite sensitive to the nature and the amount of diet consumed (Deren et al., 1967). Since monosaccharides are the end-products of carbohydrate digestion ultimately used by the cells of the body, digestion of oligo and disaccharides to monosaccharides is vital to the nutrition of the animal (Sell et al., 1989). The disaccharidases commonly studied in connection with the final stages of carbohydrate digestion include maltase (E.C. 3.2.1.20), sucrase (E.C. 3.2.1.26), lactase (E.C. 3.2.1.23) and isomaltase (E.C. 3.2.1.20). Intestinal disaccharidase activity is found mainly in mature enterocytes where absorption occurs and is virtually absent in the immature crypt cells (Alpers and Kinzie, 1973). Disaccharidases are often used as marker enzymes for mature enterocytes, and therefore could be used for assessing enterocyte maturity and possibly functional integrity in response to different biological treatments.

Organ culture experiments with enteropathogenic *E. coli* infection of rabbit ileum have revealed biochemical changes in enterocytes such as increased release of the brush border enzymes, maltase and aminopeptidase within 24 h while ultrastructure was preserved for up to 48 h (Batt et al., 1987) before changes were observed. The effects of purified bacterial endotoxin on the functional and structural integrity of the small intestine have not been well documented. However, a traumatic treatment like intestinal resection produces adaptive structural changes such as increased villi height and crypt depth in the remaining intestine (Aliaga et al., 1990; Bass et al., 1991). Preliminary studies in this laboratory indicate that ip injection of moderate doses of *E. coli* endotoxin (400 μg/kg body weight) to rats at 48 h intervals results in a drop in food intake within the first 24 h following each of the first 3 injections but not the fourth injection. It is, however, not known whether this apparent adaptation that appears to occur by about 8 d also occurs at the biochemical and structural levels in the small intestine. Therefore this study examined the effects of *E. coli (O55:B5)* endotoxin on food intake, the structure of the intestinal mucosa and on enterocyte disaccharidase activities over an 8 d period during which the *E. coli* endotoxin was injected 4 times at 48 h intervals.

## Materials and Methods:

**Materials:** Male Sprague Dawley rats weighing 130-200 g were provided food and water *ad libitum* for one week prior to starting the experiments unless stated otherwise. The animals were exposed to a 12 h light/dark cycle and were cared for in accordance with the guidelines of the Canadian Council on Animal Care. Endotoxin-free saline was obtained from Travenol Canada Inc., Mississauga ON. [$^3$H]Methylthymidine was obtained from ICN Biomedicals Inc, Irvine, CA. Endotoxin (from *E. coli (O55:B5))* and other chemicals used were obtained from Sigma Chemical Co., St. Louis, MO.

**Endotoxin Treatment**: Forty eight male Sprague Dawley rats were randomly assigned to 8 groups. Groups 1-4 each contained 7 rats which were killed after 192 h. Groups 5-8 each contained 5 rats which were killed after 24 h of treatment which was administered 24 h prior to termination of experiment, to ensure that the animals were all killed at approximately the same age. The rats were housed in individual cages and food intake was measured throughout the experimental period. Over a period of 8 days, rats in Groups 1 and 2 were injected intraperitoneally at 48 h intervals with *E. coli* endotoxin at Level 1 (100 μg/kg body weight) and Level 2 (400 μg/kg body weight), respectively. Rats in Groups 3 and 4 were injected with saline and individually pairfed with rats in Groups 1 and 2, respectively, so as to serve as pairfed controls. Rats in Groups 5, 7 and 8 were injected with saline while those in Group 6 were injected with endotoxin at Level 2. The rats in Group 5 were fasted for 24 h prior to killing to evaluate the effect of 24 h fasting on enzyme activity. Group 7 rats were pairfed with Group 6 rats. The rats in Group 8 were injected with saline and fed *ad libitum*. Intestinal tissue samples were also collected for histological examination.

**Measurement of [$^3$H]Methylthymidine Incorporation into DNA**: Twenty four hours after the last endotoxin injection, each rat in Groups 1-4 was injected via the intraperitoneal route with 0.50 ml physiological saline containing 1 mM [$^3$H]methylthymidine (150 $\mu$Ci/100 g body weight). Twenty four hours after [$^3$H]thymidine injection, (48 h after the fourth endotoxin injection) the rats were killed and the enterocytes were isolated as described below.

**Enterocyte Isolation**: The method of Reiser and Christiansen (1971) as modified by Watford et al. (1979) was used for isolating enterocytes from the small intestine. In order to estimate the overall effect of endotoxin on the function of the small intestine, enterocytes were isolated from both the jejunum and the ileum and pooled before subsampling. The whole of the small intestine was rapidly removed, weighed and it's total length measured under 5 g tension. A 90 cm segment of the small intestine beginning 5 cm below the pylorus was excised and then cut into two sections of equal length. The intestinal contents were flushed out of each section with ice-cold Krebs-Ringer bicarbonate buffer (KRB) saturated with carbogen (95% $O_2$:5% $CO_2$). A further 20 ml of gassed KRB were passed through each section to remove mucus. One end of each section was ligated with 3-0 Dexon and the sections were filled with KRB containing 1.5 mg/ml hyaluronidase and 2.5 mg/ml bovine serum albumin. The other ends were ligated and the intestinal sections were incubated in 100 ml of KRB for 15 min at 37 °C with constant agitation and gassing with carbogen. The contents were then discarded and the intestinal sections were refilled with pre-gassed KRB. Following ligation, the sections were gently tapped for 1 min with finger tips on ice-blocks covered with a polythene sheet to release enterocytes into the intestinal lumen. The KRB, containing enterocytes, from both portions were pooled in a 15 ml polythene tube and centrifuged at 1000 x g for 3 min at 4 °C. The cells were washed twice with 10 ml of gassed ice-cold KRB, centrifuged at 1000 x g for 3 min and resuspended in four volumes of KRB. Cell viability was checked by the trypan blue exclusion method (see Appendix 3). The suspension was homogenised and stored at -40 °C for subsequent assay of disaccharidases.

**Measurement of [$^3$H]thymidine Incorporation into DNA**: For Groups 1 - 4, the freshly harvested enterocytes were washed with 10 ml portions of a 1 mM solution of non-radioactive thymidine in KRB to remove free [$^3$H]thymidine. This step was repeated until radioactivity in the washes was similar to background before the cells were then homogenized in 4 volumes of KRB. The homogenate was solubilized by adding 0.4 ml of Soluene to 1 ml of homogenate and incubated overnight at room temperature. Scintillation cocktail was then added and the samples were counted in a Packard liquid scintillation analyser model 1600CA to determine the extent of [$^3$H]thymidine incorporation. The concentration of DNA in the homogenates was determined fluorometrically by a modification of the procedure of Prasad et al. (1972). This method involved hydrolysis of RNA with RNAse prior to reaction of DNA with ethidium bromide (2,7-diamino-9-phenyl-10-ethyl phenanthridinium bromide) to produce a fluorescent complex with an excitation wavelength of 365 nm and an emission wavelength of 590 nm.

**Determination of Disaccharidase Activity**: The activity of four disaccharidases, maltase, sucrase, lactase and isomaltase were determined by incubating aliquots of the enterocyte homogenate with appropriate substrates under optimum assay conditions according to the method of Dahlqvist (1964). Separate standard curves were constructed for samples assayed on different days. The glucose liberated was measured by the method of Trinder (1969). Protein was measured by a modification of Lowry's method developed by Hartree (1972).

**Statistical Analysis**: The results from all groups were analysed by one-way analysis of variance, and multiple comparison of means of Groups 1-4 was by Student-Newman-Keuls test. Differences between treatment and pair fed control means were evaluated by Student's t-test.

**Results**:

**Effect of Endotoxin on Food Intake**: Food intake, during the initial 24 h period following the first endotoxin injection was reduced in rats receiving endotoxin at Level 1 ($P<0.05$) and Level 2 ($P<0.01$) compared to the *ad libitum* fed control group injected with saline. Saline injection did not affect the food intake of the *ad libitum* fed rats (Group 8). Therefore, the average daily intake throughout the experimental period was plotted as a single value in Fig. IV-1. Food intake was not reduced following subsequent endotoxin injections in rats treated at Level 1. However, food intake was reduced during the initial 24 h period ($P<0.05$)

in Level 2 treated rats following the second and third endotoxin injections (See Fig. IV-1). There was no reduction in food intake following the fourth endotoxin injection at Level 2. Food intake was not reduced during the period between 24 h and 48 h post endotoxin injection at any time during the experiment. Even though the second and third endotoxin challenges at Level 2 still produced lower ($P<0.05$) food intake compared to saline injected *ad libitum* fed rats during the initial 24 h following injection, the animals showed a gradual adaptation to the endotoxin since food intake was less reduced with each subsequent endotoxin challenge. On a subjective basis, the endotoxin treated rats also appeared more lively than they were during the initial 24 h period following the first challenge.

**Intestinal Size, Incorporation of [$^3$H]Thymidine into DNA and Mucosal Histology**: Neither the weight nor the length of the intestine was affected by endotoxin (Table IV-1). Endotoxin treatment resulted in an increase in the extent of incorporation of [$^3$H]thymidine radioactivity into DNA above that measured in pairfed control animals (Table IV-2) during the last 24 h of the 192 h treated groups. The increases were 38% ($P<0.05$) at Level 1 and 86% ($P<0.01$) at Level 2 of endotoxin administration. Endotoxin did not affect tissue DNA concentrations. Histological sections of the small intestine from the endotoxin treated animals showed relatively shorter villi with distorted architecture than the sections from the controls as shown in Plate IV-1.

**Disaccharidase Activity**: Maltase activity in enterocytes from 24 h fasted rats was lower (P<0.05) than the activity in enterocytes from saline injected *ad libitum* fed rats. On the other hand, maltase activity in enterocytes from rats injected with endotoxin at either Level 1 or Level 2 was not significantly (P>0.05) different from their pairfed saline injected control rats sacrificed 24 h or 192 h after the initial endotoxin injection (Fig. IV-2). The activity of this enzyme in enterocytes from endotoxin treated groups and their pairfed controls was significantly lower (P<0.05 and P<0.01 for Levels 1 & 2, respectively) than in enterocytes from the saline injected, *ad libitum* fed rats. Maltase activity in enterocytes from rats treated for 192 h with 400 μg endotoxin/kg body weight (Level 2) was reduced to about 45% of the activity found in enterocytes from rats that were injected with saline and fed *ad libitum*.

Sucrase activity in enterocytes from 24 h fasted rats and from rats 24 h after the first endotoxin injection were similar and lower (P<0.05) than the activity found in enterocytes from saline injected, *ad libitum* fed rats. After 192 h sucrase activity in enterocytes isolated from endotoxin treated rats was not significantly (P<0.05) different from that in enterocytes isolated from their pairfed controls (Fig. 3) but was significantly lower than the activity in enterocytes from saline injected rats fed *ad libitum*. Sucrase activity in the enterocytes from Level 2 endotoxin treated (400 μg/kg body weight) rats over 192 h period, was reduced to 47% of the activity in enterocytes from rats injected with saline and fed *ad libitum*.

Endotoxin had the greatest effect on lactase activity compared to the other three disaccharidases measured in the enterocytes. Lactase was the only enzyme whose activity in endotoxin treated animals was lower than it's activity in saline injected, pairfed control rats. Compared to pair-fed controls, enterocyte lactase activity was significantly decreased by the endotoxin treatment at Level 2 for 192 h, but the decrease was not statistically significant ($P>0.05$) at Level 1 (Fig. IV-4). Lactase activity in enterocytes from endotoxin treated animals sacrificed at 24 h or 192 h was much lower than that found in enterocytes from rats that were injected with saline and fed *ad libitum*. Lactase activity from endotoxin treated Level 2 rats at the end of the 192 h experimental period was reduced to about 20% of the activity in enterocytes from rats that were fed *ad libitum*. Fasting (24 h) also decreased ($P<0.05$) enterocyte lactase activity.

The activity of isomaltase was not affected ($P>0.05$) by fasting or endotoxin (Fig. IV-5). Isomaltase had the lowest activity compared to the other disaccharidases studied.

## Discussion:

The present studies confirmed our preliminary studies that intraperitoneal administration of *E. coli* endotoxin at 400 μg/kg body weight reduces food intake only during the initial 24 h following injection. During the second 24 h period after endotoxin injection, food intake was no longer statistically ($P>0.05$) different from the food intake of the saline injected control. Therefore, to maintain observable effects of endotoxin over the duration of the 8 day treatment period, endotoxin was administered at four 48 h intervals. The drastic drop in feed intake recorded in this experiment in response to the first challenge with endotoxin followed by substantial amelioration in response to subsequent challenges, demonstrates the ability of rats to adapt to low or sublethal doses of endotoxin. Jepson et al. (1986) have reported a similar pattern of food intake in rats following *E. coli O127:B8* endotoxin injection.

Incorporation of [$^3$H]thymidine into enterocyte DNA was increased by the endotoxin treatment without affecting DNA concentrations. The increase in the incorporation of thymidine radioactivity into the enterocytes is indicative of an increase in the rate of cell replication. Since the size of the intestine of the endotoxin treated rats was not different from their pair fed controls, the increase in [$^3$H]thymidine radioactivity per mg of DNA may be explained by an increase in the rate of enterocyte turnover. Since no information was obtained in this study on cell cycle time, growth fraction or cell migration rates it is hard to determine precisely whether the increase in [$^3$H]thymidine incorporation is a reflection of increased enterocyte turnover rates. However, an increase in cell replication without a change in the rate of cell breakdown would have produced a net increase in the size of the intestine but intestinal size expressed as a fraction of the body weight did not differ between the control and the endotoxin treated rats (Table IV-2). It is therefore possible that enterocyte turnover rates may have been increased. An increase in turnover rate would mean that a higher proportion of the enterocytes were undergoing replacement by newly synthesized enterocytes between the time the endotoxin was administered and the time the rats were killed. The presence of Staphylococcal enterotoxin has been said to increase the migration rate of enterocytes from the crypt to the tip of the villus in rat (Alpers and Kinzie, 1973). The intestinal mucosa is plastic and responds to a change in cell turnover by altering its gross villus architecture, with the normal finger-like villi altering their shape to leafed or spaded configurations (Snipes, 1967). A decrease in villus height to a leaf-like structure in response to E. coli endotoxin was also observed in rats in a preliminary study in which rats were injected with endotoxin at 400 μg/kg body weight (See Plates IV-1a & IV-1b). A decrease in villus height and or an increase in enterocyte migration rate, would result in a much shorter life span for the enterocytes. The procedure used for isolating the enterocytes in the present study mainly removes mature cells at the upper part of the villus (Reiser and Christiansen, 1971). Therefore the higher [$^3$H]thymidine radioactivity found in enterocytes isolated from endotoxin treated rats indicates that a higher proportion of the enterocytes from endotoxin treated animals were synthesized post [$^3$H]thymidine administration.

The enterocyte maltase and sucrase activities of endotoxin treated rats were found not to differ significantly from the values obtained from pairfed control rats ( Fig. IV-2 ) but were significantly lower than the activities found in the saline injected *ad libitum* fed rats. The pattern of adaptation in food intake in response to endotoxin administration appears to be different from that of the disaccharidases because the activities of the disaccharidases in endotoxin treated rats were still lower than the activities in the saline injected *ad libitum* fed rats even after the food intake had almost returned to normal. In other words, the recovery phase in terms of food intake is faster than that of the disaccharidases. A 20% decrease in food intake and a significant decrease ($P<0.05$) in the activity of maltase and sucrase in the jejunum and distal ileum of infected and pair fed control have been reported in mice following murine Giardiasis (Buret et al., 1990). In their study, complete recovery in the activity of these enzymes was only reported on day 24 post infection even though the 20% reduction in food intake only occurred during the first 12 days of the infection. Malnutrition has also been shown to decreases the activities of maltase and lactase (Young et al., 1987) while sucrase activity is enhanced by sucrose feeding (Deren et al., 1967). A dietary component such as pectin has also been shown to enhance the activities of intestinal maltase, sucrase and lactase (Koruda et al., 1988). However, dietary composition was not a variable in this experiment as all the animals ate the same diet.

Lactase was the only disaccharidase measured for which it's activity was significantly lower ($P<0.05$) in enterocytes from endotoxin treated rats than in enterocytes from their pair fed controls. The reduction in the activity of this intestinal disaccharidase in the endotoxin treated animals is a composite of the direct effect of endotoxin and the effect of reduced food intake. A decrease only in lactase activity in patients with mild mucosal lesions has been documented (Langman and Rowland, 1990). On the other hand pathological lesions had to be moderate or severe before maltase and sucrase activities were lowered. It is not clear why lactase appears to be more responsive than the other disaccharidases but it may be related to differences in turnover rates of the enzymes. Low lactase activity is not likely to pose serious problems in adult animals since lactose is not normally a significant component of their diets. However, these findings suggest that severe endotoxemia could have drastic effects on young suckling animals. It is not possible to tell the nature of inhibition of enzyme activities (ie whether it is $K_M$ or $V_{max}$ that is affected) by endotoxin because the study did not involve determination of kinetic parameters.

Neither endotoxin treatment nor fasting had a significant effect on isomaltase activity. This observation may be indicative of the limited importance of isomaltose hydrolysis in carbohydrate digestion.

In conclusion, systemic administration of endotoxin resulted in increased apparent enterocyte turnover rate, decreased food intake, and food intake related changes in disaccharidase activities. However, complete recovery in food intake did not result in complete recovery in enzyme activity 48 h following endotoxin injection suggesting differences in the pattern of adaptation.

Table IV-1: Effect of E coli endotoxin on intestinal size.

Rats were injected with endotoxin at 48 h intervals and killed 48 h after the last injection.

Values are presented as means ± SEM. n=7

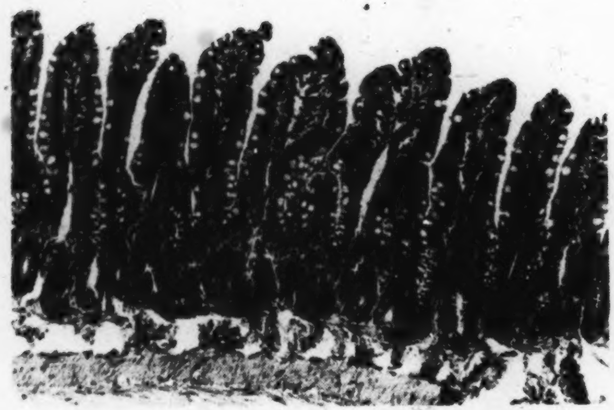
| | Intestinal Size | | | |
|---|---|---|---|---|
| Treatment level | Control | | Endotoxin | |
| | Weight | Length | Weight | Length |
| µg/kg b. weight | (% body weight) | (cm) | (% body weight) | (cm) |
| 100 | 3.93 ± 0.10 | 111.5 ± 2.1 | 3.76 ± 0.13 | 107.7 ± 2.6 |
| 400 | 3.74 ± 0.18 | 109.0 ± 1.7 | 3.70 ± 0.08 | 110.4 ± 2.0 |

Table IV-2: Effect of E coli endotoxin on [$^3$H]thymidine incorporation into DNA. The rats were killed 48 h after the last endotoxin injection (192 h after the first injection). Values are presented as means ± SEM. * significantly ($P<0.05$) different from pair-fed control, ** significantly ($P<0.01$) different from pair-fed control. n=7

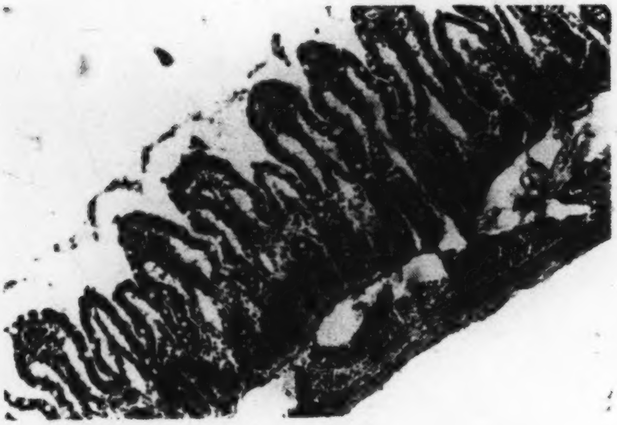
| Treatment level | Pair Fed Control | | Endotoxin | |
|---|---|---|---|---|
| | Incorporation | DNA concentration | Incorporation | DNA concentration |
| µg/kg b. weight | (dpm/mg DNA) | (µg/mg protein) | (dpm/mg DNA) | (µg/mg protein) |
| 100 | 2217 ± 49 | 64.4 ± 4.4 | 3064 ± $89^{*}$ | 70.3 ± 3.7 |
| 400 | 2273 ± 51 | 67.6 ± 5.5 | 4230 ± $76^{**}$ | 62.6 ± 5.5 |

**Plate IV-1 Effect E coli Endotoxin on Villi Structure.**

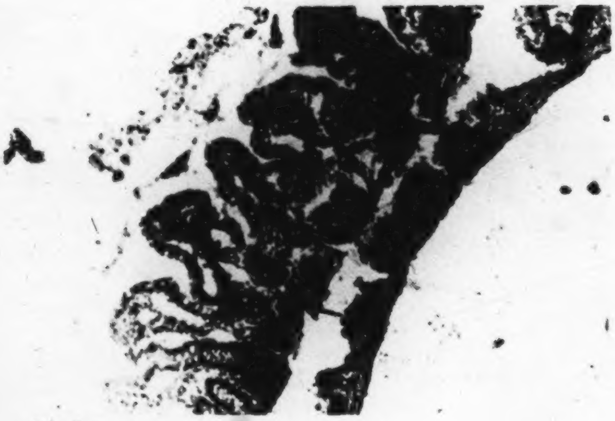
(a) A typical structure of intestinal mucosa from a control rat, (b) A typical structure of the intestinal mucosa from an endotoxin treated rat, (c) A very severe case found in one of the endotoxin treated rats. Slide magnification: x 250.



IV-1a



IV-1b



IV-1c

Fig. IV-1. Effect of Endotoxin on Food Intake

Results are plotted as means and standard errors.

☐, Average daily food intake of saline injected control rat.

.■ Group treated with Level 1 endotoxin (100 μg/kg body weight).

.▨ Group treated with Level 2 endotoxin (400 μg/kg body weight).

Injections were carried out at on days o, 2, 4, and 6 immediately after food intake and body weight measurements had been taken.

Statistical significance: * different ($P<0.05$) from ad libitum food intake, ** different ($P<0.01$) from ad libitum food intake. $n=5$ or 7

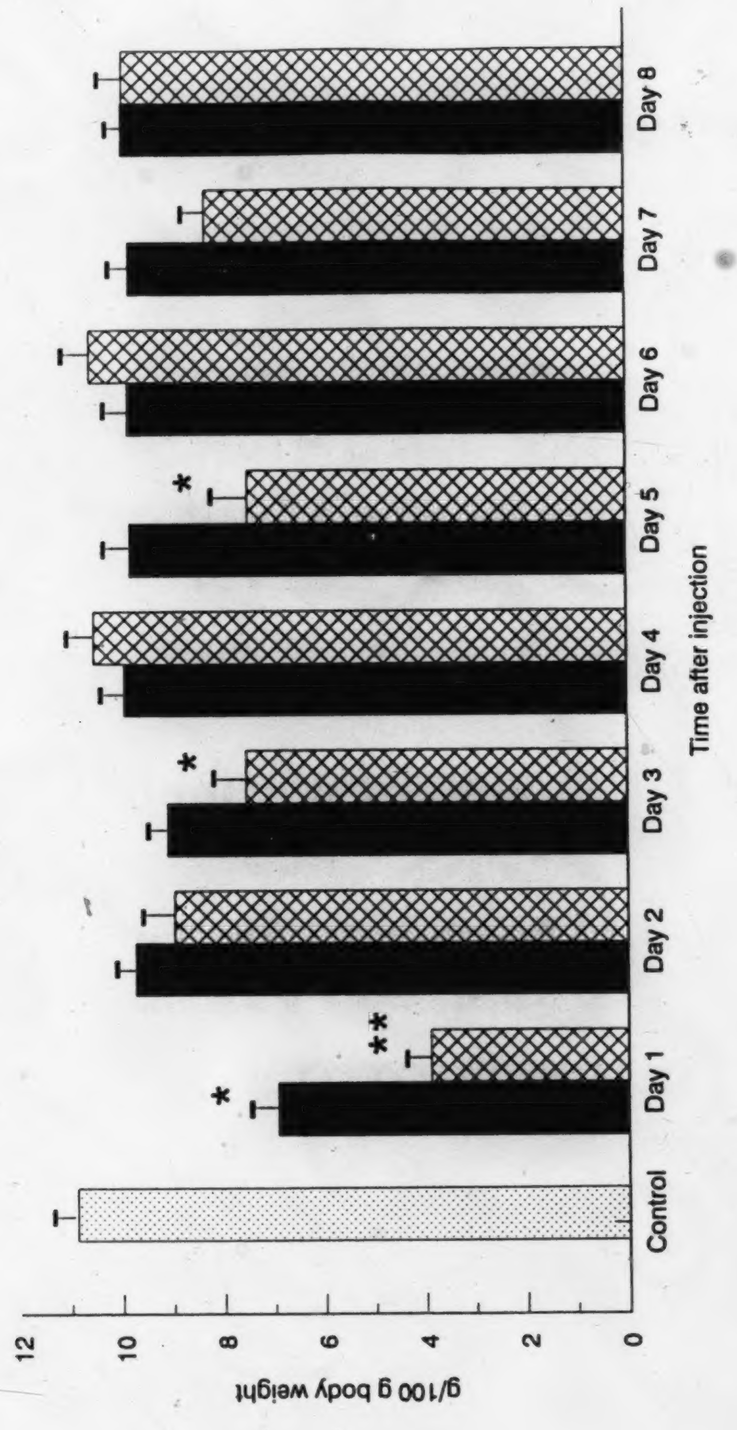

g/100 g body weight
0
2
4
6
8
10
12
Control
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Time after injection
*
**
*
*

Fig. IV-2. Effect of Endotoxin on Maltase Activity.

Results are plotted as means and standard errors.

■, Saline injected, ad libitum fed control, killed 24 h after injection.

▨, After 24 h fast.

☒, 24 h after first endotoxin injection.

⧅, Pair fed control 24 h after saline injection.

▩, Day 8 following 4 successive endotoxin injections at intervals of 48 h.

□, Pair fed control on day 8 following 4 successive saline injections at intervals of 48 h.

Statistical significance: * different ($P<0.05$) from ad libitum fed control, ** different ($P<0.01$) from ad libitum fed control. Bars bearing identical markers are not statistically different from each other. $n=5$ or 7.

Maltase

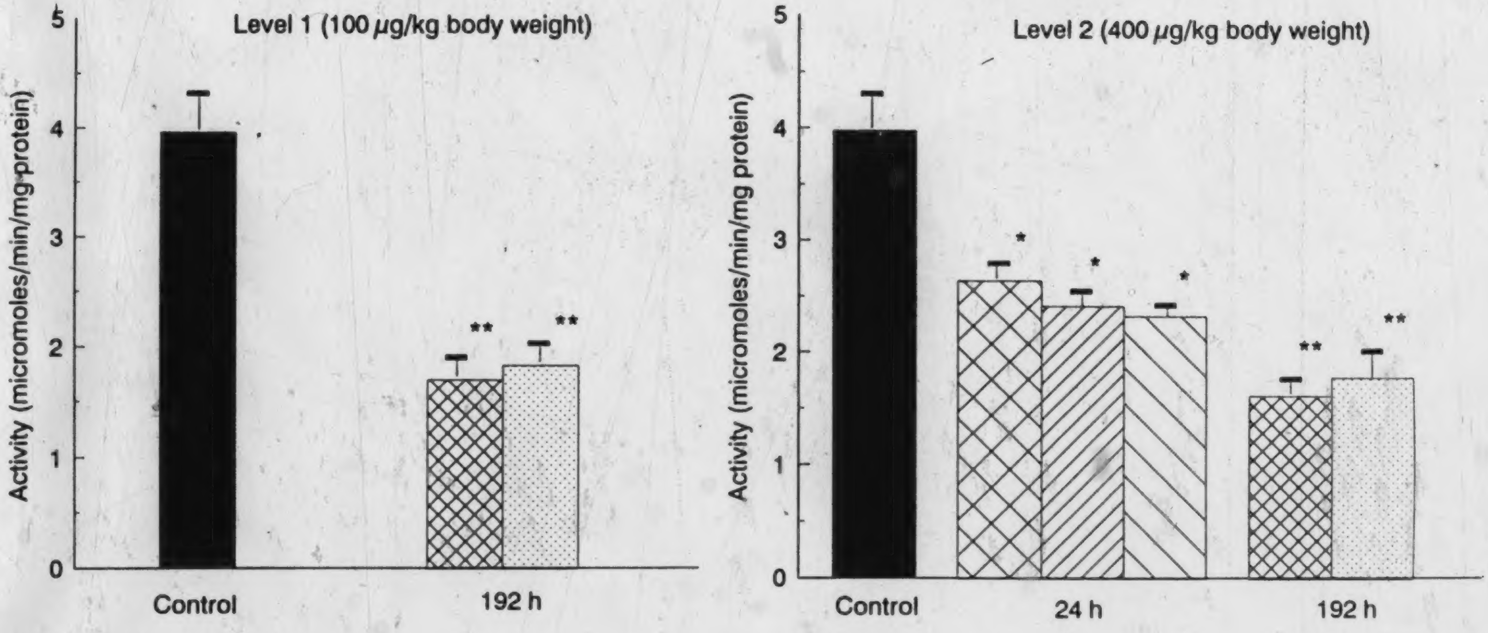

Level 1 (100 µg/kg body weight)
Activity (micromoles/min/mg protein)
0
1
2
3
4
5
Control
192 h
**
**
Level 2 (400 µg/kg body weight)
Activity (micromoles/min/mg protein)
0
1
2
3
4
5
Control
24 h
192 h
*
*
*
**
**

Fig. IV-3. Effect of Endotoxin on Sucrase Activity.

Results are plotted as means and standard errors.

■, Saline injected, ad libitum fed control, killed 24 h after injection.

▨, After 24 h fast.

⊠, 24 h after first endotoxin injection.

⧅, Pair fed control 24 h after saline injection.

▩, Day 8 following 4 successive endotoxin injections at intervals of 48 h.

☐, Pair fed control on day 8 following 4 successive saline injections
at intervals of 48 h.

Statistical significance: * different ($P<0.05$) from ad libitum fed control, ** different ($P<0.01$) from ad libitum fed control. Bars bearing identical markers are not statistically different from each other. $n=5$ or 7.

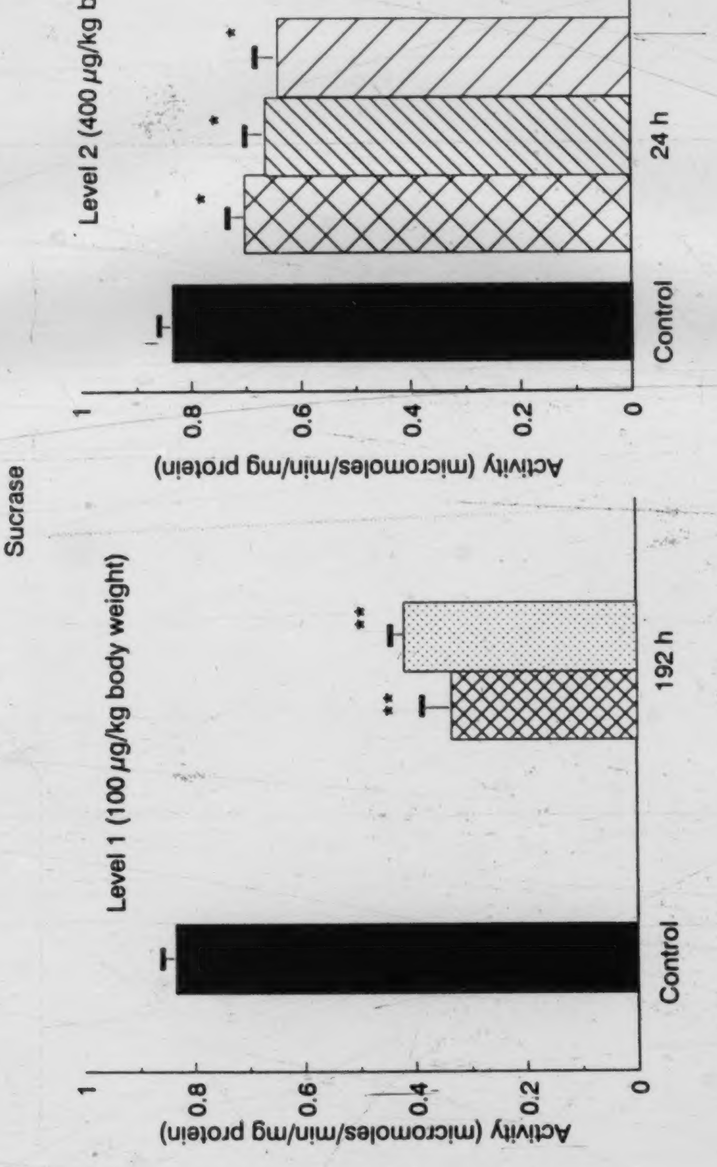

Sucrase
Level 1 (100 µg/kg body weight)
Level 2 (400 µg/kg body weight)
Activity (micromoles/min/mg protein)
0
0.2
0.4
0.6
0.8
1
Control
24 h
192 h
*
**

Fig. IV-4. Effect of Endotoxin on Lactase Activity.

Results are plotted as means and standard errors.

■, Saline injected, ad libitum fed control, killed 24 h after injection.

▨, After 24 h fast.

⊠, 24 h after first endotoxin injection.

▧, Pair fed control 24 h after saline injection.

▩, Day 8 following 4 successive endotoxin injections at intervals of 48 h.

□, Pair fed control on day 8 following 4 successive saline injections at intervals of 48 h.

Statistical significance: * different ($P<0.05$) from ad libitum fed control, ** different ($P<0.01$) from ad libitum fed control, *** different ($P<0.001$) from ad libitum fed control. Bars bearing identical markers are not statistically different from each other. $n=5$ or 7

Lactase

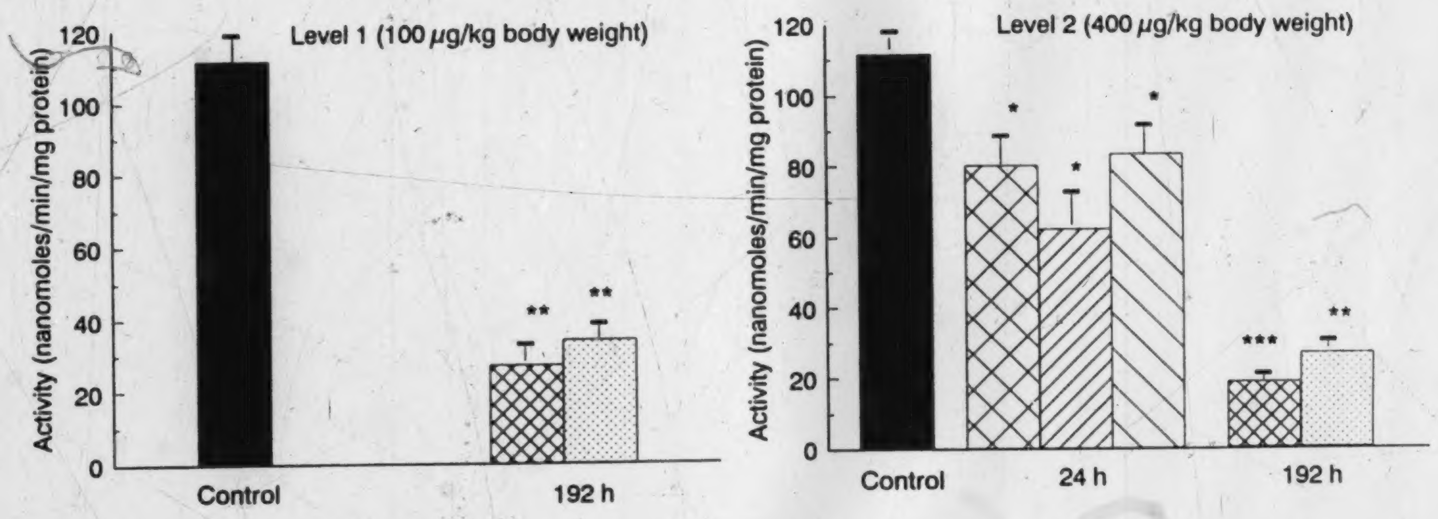

Level 1 (100 μg/kg body weight)
Activity (nanomoles/min/mg protein)
120
100
80
60
40
20
0
Control
192 h
**
**
Level 2 (400 μg/kg body weight)
Activity (nanomoles/min/mg protein)
120
100
80
60
40
20
0
Control
24 h
192 h
*
*
*
***
**

Fig. IV-5. Effect of Endotoxin on Isomaltase Activity.

Results are plotted as means and standard errors.

■, Saline injected, ad libitum fed control, killed 24 h after injection.

▨, After 24 h fast.

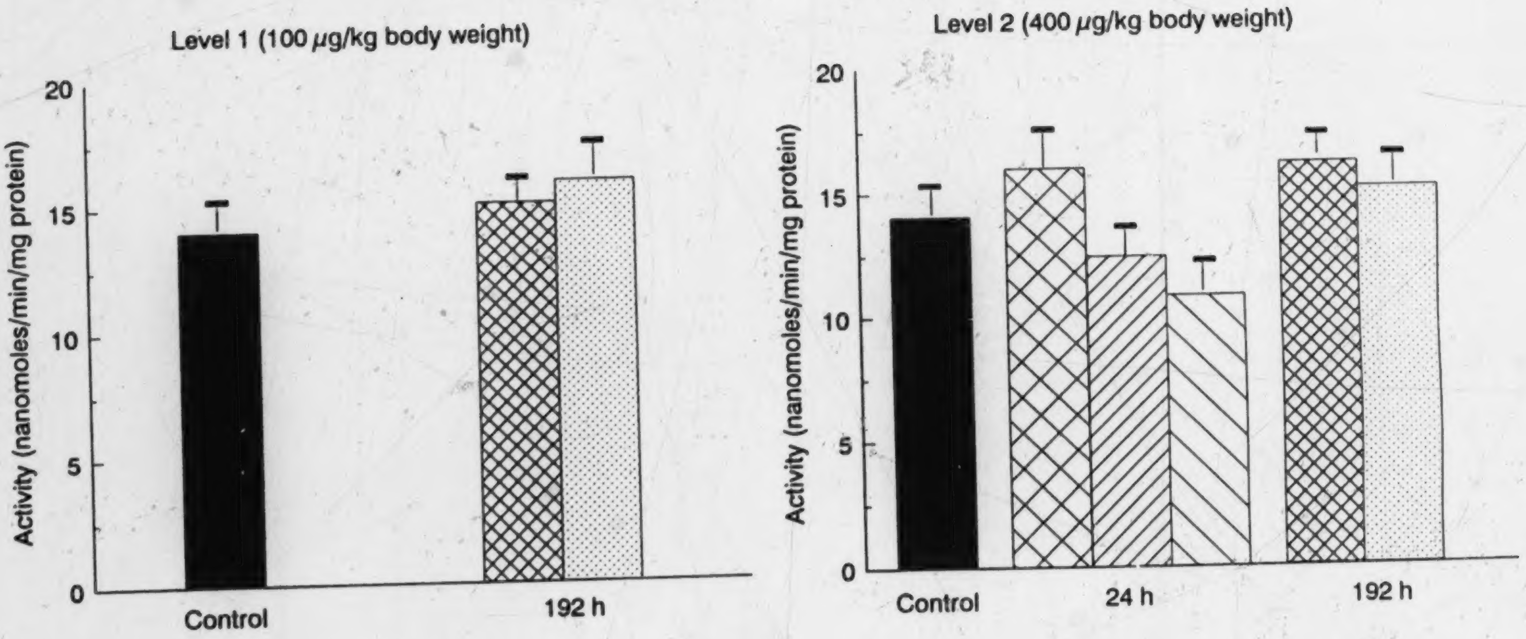
⊠, 24 h after first endotoxin injection.

⧄, Pair fed control 24 h after saline injection.

▩, Day 8 following 4 successive endotoxin injections at intervals of 48 h.

░, Pair fed control on day 8 following 4 successive saline injections at intervals of 48 h.

There was no significant difference ($P>0.05$) in activity. $n=5$ or 7



Isomaltase

Level 1 (100 μg/kg body weight)
Activity (nanomoles/min/mg protein)
20
15
10
5
0
Control
192 h
Level 2 (400 μg/kg body weight)
Activity (nanomoles/min/mg protein)
20
15
10
5
0
Control
24 h
192 h

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# V Ameliorating Effects of Dietary Glutamine Supplementation on the Endotoxic Response in Rats

## Introduction:

Nutritional support programs for patients focus mainly on supplying adequate amounts of calories and essential nutrients including amino acids and vitamins. Glutamine has not been classified as an essential amino acid (Rose, 1938; Souba et al., 1990a) but recent advances in clinical nutrition have led to suggestions that it may become essential under some clinical settings (O'Dwyer et al., 1989; Smith and Wilmore, 1990). The major site of glutamine production appears to be the skeletal musculature (Felig, 1975). It is well established that there is a dramatic decline in skeletal muscle and/or blood free glutamine concentrations during infection (Askanazi et al., 1980; Kapadia et al., 1985; Wolfe et al., 1976; Milewski et al., 1982), injury (Aulick and Wilmore, 1979; Caldwell, 1989; Albina et al., 1986; Rennie et al., 1989), surgery (Vinnars et al., 1975; Kapadia et al., 1985; Hartmann and Plauth, 1989), burns (Ardawi, 1988) and other forms of trauma to the extent that glutamine may become limiting.

Relative to other tissues in the body, the intestinal mucosa has one of the highest rates of cell turnover (Alpers and Kinze, 1973; O'Dwyer et al., 1989). Maintenance of normal intestinal architecture and function requires a constant supply of energy and biological modulators. In contrast to the cells of most tissues in the body, the enterocytes derive a major portion of their energy requirement from glutamine. A possibility therefore exists that glutamine may be an important modulator of intestinal function. Stressful conditions such as injury, surgery (Souba and Wilmore, 1983), infection (Wilmore et al., 1988) or glucocorticoid administration (Souba et al., 1985) give rise to increased intestinal glutamine demand. Current evidence indicates that there is not a concomitant increase in glutamine synthesis sufficient to match the increased rate of utilization under these conditions (Souba et al., 1990a). Therefore a progressive decrease in muscle free glutamine concentrations is often observed during these catabolic conditions.

Experiments with rats maintained on defined formular diets with adequate amounts of calories but lacking in glutamine and fiber have shown that glutamine and fiber play significant roles in the maintenance of bowel mass and intestinal barrier integrity (Barber et al., 1990). Rats maintained on these defined diets showed an increased incidence of bacterial translocation, hepatic steatosis and susceptibility to endotoxin.

Radiation injury to the intestinal mucosa has been associated with destruction of proliferative crypt cells, decreased villi height and ulceration or necrosis of the gut epithelium (Klimberg et al., 1990a; Berthrong, 1986). Oral glutamine administration accelerates the healing of the small intestine (Klimberg et al., 1990a) and prophylactic benefits have been derived from glutamine against radiation injury (Klimberg et al., 1990b). Supplementation of parenteral formulars with glutamine has been associated with preservation of the normal jejunal architecture

(Barber et al., 1990), decreased bacterial translocation and improved survival in experimental enterocolitis (Fox et al., 1988). It is not known however, whether dietary supplementation with glutamine can prevent or ameliorate these adverse developments. It is well documented that only the mature enterocytes at the upper part of the villus have significant absorptive functions, while the undifferentiated cells in the crypt region have no absorptive function (Madara and Trier, 1987). Any agent that reduces the villi height, villi width or villi density will ultimately impair the absorptive process while any agent that damages the proliferative crypt cells is likely to impair the replacement of old enterocytes that slough off from the villi tips and ultimately lead to mucosal atrophy with the attendant alteration in gut permeability to xenobiotics. In chapter IV it was reported that endotoxin decreases villi height and alters the typical villi architecture. It is not known whether dietary glutamine supplementation can ameliorate these adverse responses to endotoxin.

Any nutritional intervention aimed at preventing or reducing damage is likely to yield more benefits than treatments aimed at promoting the healing process following damage. Nutritional supplementation could be accomplished via both the enteral and parenteral routes. Where possible, nutritional supplementation through the enteral route is much easier and physiologically more beneficial (Johnson et al., 1975; Hughes and Dowling, 1980) especially in farm animals where nutritional supplementation through the parenteral route could prove technically difficult. Nutritional supplementation through the enteral route could be accomplished either by supplying the required nutrient(s) in solution or in solid form. In the case of glutamine, supplementation in aqueous form at physiological pH as free glutamine is not feasible because glutamine is unstable in aqueous solution over a prolonged length of time. The model developed in this study is one in which glutamine was incorporated homogenously into the feed which was then fed to the rats with the objective of examining whether glutamine plays any ameliorating role in the course of events that are triggered by exposure to endotoxin.

**Materials and Methods:**

**Materials:** Male Sprague Dawley rats were obtained one week prior to starting the experiment, provided food and water *ad libitum* in individual metabolic cages and exposed to a 12 h light/dark cycle. Animal care and treatment administration were in accordance with the guidelines of the Canadian Council of Animal Care. At the time the treatments were administered, the rats weighed between 260-340 g. Endotoxin-free saline was obtained from Travenol Canada Inc., Mississauga ON. Endotoxin (from *E. coli (O55:B5))* and other chemicals used were obtained from Sigma Chemical Co., St. Louis MO.

**Preparation of the Diets:** The acid stable amino acid composition of the rat chow based diets was determined by high performance liquid chromatography (Jones and Gilligan, 1983) after acid hydrolysis with 6 N HCl at 110 °C for 24 h. The diets were based on rat chow because it is the regular diet of rats, but its glutamine content may not be adequate for the rat during pathological conditions like infection or endotoxemia. Glutamine and asparagine were determined by HPLC after digestion with carboxypeptidase A. Since complete digestion of the diet by the enzyme could not be guaranteed, the concentration of glutamine in the rat chow was estimated by using the ratio of the areas of glutamine:glutamic acid in the carboxypeptidase digest to calculate the amount of glutamic acid that arose from glutamine in the acid digest. The same was done for estimating the concentrations of asparagine and aspartic acid. The tryptophan content of the rat chow was also estimated in the carboxypeptidase digest. Blank tubes containing the enzyme alone were included for correcting for possible contamination from the enzyme. The rat chow contained 0.89% (w/w) glutamine. Supplementation was carried out at the 4% level to provide a 5 fold increase in glutamine. The glutamine supplemented (4%, w/w) diet was prepared according to the following procedure. Nine hundred and sixty grams of feed were sprayed with an aqueous solution containing 40 g glutamine from a fine jet while vigorously mixing the feed in a feed mixer. This was repeated 4 times to obtain about 5 kg of damp feed which was pooled together, spread thinly on wide aluminium trays and dried in a warm room maintained at 37 °C. The basal diet was made isonitrogenous with the glutamine supplemented diet by the addition of glycine (2 moles of glycine for each mole of glutamine in the glutamine supplemented diet) using a similar procedure.

**Endotoxin Injection and Sample Collection:** A total of 24 male Sprague Dawley rats housed in individual cages were randomly assigned to 4 groups of 6 rats per group. Groups 1 and 4 rats were fed the basal diet while groups 2 and 3 were fed the glutamine supplemented diet. Body weight and food intake were recorded daily. After 4 days on these diets, blood was collected from the tail vein of rats in Groups 2 and 4 (the Groups to be injected with endotoxin) into heparinized tubes then the rats in Groups 1 and 3 were injected intraperitoneally (ip) with saline and those in Groups 2 and 4 were injected with endotoxin (400 μg/kg) in saline. Blood was collected from the tail vein of rats in Groups 2 and 4 at 24 h and 48 h after endotoxin injection. Saline and endotoxin injections were repeated in the respective groups at 48 h, 96 h and 144 h after the initial injection. The rats in each of the four groups were killed by decapitation at 192 h after the initial injection of saline or endotoxin. Blood was collected in heparinized tubes for plasma isolation. Gastrocnemius and soleus muscles were dissected, weighed, placed inside aluminium foil and frozen immediately in liquid nitrogen. Two 1 cm segments of the small intestine were removed from the jejunum, quickly washed in cold Krebs-Ringer buffer and preserved in buffered formalin for histological study. Plasma was isolated from the blood by centrifugation at 2500 x g, deproteinized with trichloroacetic acid and analysed for glutamine by high performance liquid chromatography.

The separation of glutamine from other amino acids and its quantification was accomplished with a model 5000 Varian high performance liquid chromatograph and a Varian Fluorichrome detector with excitation and emission wavelengths of 340 nm and 450 nm, respectively (see Appendix 2 for details). The muscle samples were homogenized in 2% TCA and also analysed by HPLC for free glutamine.

The jejunal segments were fixed at 4 °C for 48 h in 4% buffered formalin then dehydrated in ascending grades of alcohol and cleared in xylene as outlined in Appendix 5. The segments were embedded in paraffin wax and 5 μm sections were cut on a microtome and stained in haematoxylin and eosin (H. & E.). Villi height and crypt depth were measured in each section in triplicate with the aid of a stage micrometer and a mean value for each animal.

**Statistical Analysis**: Results were analysed by analysis of variance. Where the treatment effect was significant, differences between the treatment mean and the control mean were tested by Student's t test.

**Results**:

**Effect of Endotoxin and Glutamine Supplementation on Body Weight**: Intraperitoneal injection of rats with *E. coli (055:B5)* endotoxin caused a drop ($P<0.05$) in 24 h body weight (Fig. V-1). As shown in Figure. V-1 the lowest body weight throughout the period of the experiment was recorded among the endotoxin treated rats 24 h following the initial endotoxin injection. Improvement over this initial 24 h weight was noticed during subsequent endotoxin challenges but body weight of endotoxin injected rats did not catch up with the saline injected controls. The body weights of rats in the two groups that were injected with endotoxin were less than those for rats injected with saline and remained lower than the pre-injection body weight throughout the 8 day period. Glutamine supplementation had no effect on body weight in either the control and endotoxin treated animals.

**Effect of Endotoxin and Glutamine Supplementation on Food Intake**: Food intake by the endotoxin injected animals was lower ($P<0.01$) during the first 24 h following the initial injection than in the saline injected control animals (Fig. V-2). Subsequent endotoxin injections at 48 and 96 h resulted in smaller but significant ($P<0.05$) decreases in food intake. Food intake was not reduced during the second 24 h period following endotoxin injection at any time during the experiment. Glutamine supplementation of the diet had no effect on food intake in either the treated or control groups.

**Effect of Endotoxin and Glutamine Supplementation on Plasma Free Glutamine Concentration**: A comparison of the endotoxin injected versus the saline injected groups fed the basal diet showed that endotoxin injection resulted in a significant decrease ($P<0.05$) in plasma free glutamine concentration (Table V-3). Dietary supplementation with glutamine did not restore the plasma concentration of glutamine during the first 24 h after the initial endotoxin injection (Table V-2). Glutamine supplementation prevented the endotoxin-induced decrease in plasma free glutamine concentration that remained 8 days after the initial endotoxin injection (Table V-3). In the absence of endotoxin, dietary glutamine supplementation did not affect plasma free glutamine concentrations (Table V-3).

**Effect of Endotoxin and Glutamine Supplementation on Intramuscular Free Glutamine Concentration:** Endotoxin injection over a period of 192 h resulted in a decrease (P<0.05) in intramuscular free glutamine concentration in the gastrocnemius but not in the soleus muscles (Table V-4). Dietary glutamine supplementation alleviated the endotoxin-induced decrease in the gastrocnemius free glutamine concentration. In the absence of endotoxin, glutamine supplementation did not significantly (P>0.05) affect muscle free glutamine concentration.

**Effect of Endotoxin and Glutamine Supplementation on Jejunal Mucosa Histology:** Rats injected with endotoxin and fed the basal diet had a significantly (P<0.01) lower villi height and higher (P<0.01) crypt depth compared to the saline injected group fed the basal diet (Table V-5). Mean villi height and crypt depth of rats injected with endotoxin and fed the glutamine supplemented diet were not significantly (P>0.05) different from the rats injected with saline and fed the basal diet. Similarly, glutamine supplementation in the absence of endotoxin had no effect on either villi height or crypt depth (Table V-5). The villi in endotoxin treated animals fed the basal diet apart from being shorter than the villi in control animals, did not posses the typical finger-like appearance. Glutamine supplementation preserved the normal features of the mucosa as well as the normal villi height and crypt depth (Plates V-1a, V-1b & V-1c.)

**Discussion:**

The sharp drop in body weight following the initial 24 h post endotoxin injection coincides with the drastic reduction in food intake and may be largely due to the reduction in gut fill. During the time period from 24 h to 48 h post endotoxin injection considerable recovery in both body weight and food intake was noted. As the animals gradually adapted to the effects of the endotoxin, there was a parallel recovery in body weight and in food intake. By day 8, gut fill may only account for a small proportion of the differences in body weights between the control and the endotoxin treated rats. The absence of a statistically significant effect of endotoxin during the second 24 h after injection may result from the detoxifying action of the liver.

Dietary supplementation with glutamine even at this relatively high level (about 5 times the level found in normal rat chow diet) did not elicit adverse effects or symptoms in the animals, using food intake and body weight changes as performance indices. A comparison of the saline injected group fed the basal control diet with the saline injected group fed the glutamine supplemented diet shows that in healthy animals no significant benefit is derived from additional glutamine supplementation. In contrast, addition of glutamine to a glutamine free parenteral diet has been reported to improve nitrogen retention (O'Dwyer et al., 1989). Since glutamine supplementation did not maintain body weight of endotoxin treated rats at control values in the present study, it is apparent that other factors in addition to glutamine availability are involved in regulating body weight under these conditions.

In endotoxin injected rats, glutamine supplementation had a positive influence on plasma glutamine and intramuscular free glutamine concentrations in the gastrocnemius muscle. The plasma glutamine pool is supplied mainly by the diet, by synthesis in the skeletal muscle, and by protein breakdown. In a recent report, Souba et al., (1990b) have indicated that the lung may also be an important site for glutamine production. Most of the glutamine released from the skeletal musculature is synthesized *de novo* by the enzyme glutamine synthetase. The fact that plasma glutamine concentrations in the saline injected group fed the glutamine supplemented diet did not differ from those in the group injected with saline and fed the basal control diet means that the body can readily down regulate the activity of glutamine synthetase or step up the metabolic reactions that consume glutamine when the dietary supply increases. In fact, O'Dwyer et al., (1989) have reported lower glutamine synthetase activity in the jejunum of rats fed a glutamine supplemented diet than in rats fed glutamine deficient diets.

In the endotoxin injected rats, like other stressful conditions, the glutamine demand seems to exceed the threshold of glutamine synthetase warranting additional dietary glutamine in order to maintain plasma glutamine concentrations. The inability of the glutamine supplemented diet to maintain normal plasma concentration during the first 24 h after injection may be due to the drastic reduction in food intake. However, the control plasma glutamine concentration was attained 48 h after the first endotoxin injection. This period corresponds to the time when food intake had returned close to the pre-injection intake values.

The intestinal mucosa has one of the highest turnover rates among tissues in the body. Like all rapidly turning over cells, the cells of the intestinal mucosa have a high requirement for glutamine (Smith, 1990) which is channelled into 3 areas. Quantitatively, the first major function is to meet part of the energy needs of cells, while the second is to provide glutamine for protein synthesis and lastly, to provide nitrogen for nucleotide biosynthesis which is elevated in rapidly replicating cells. Therefore any agent that increases the turnover rate or shortens the life span of the cells will increase the demand for glutamine. Since glutamine supplementation of the diet consumed by the endotoxin treated rats ameliorated the decrease in villi height and the increase in crypt depth, there is evidence to suggest that endotoxin increased the glutamine requirements of the animal possibly by increasing the sloughing off rate of the enterocytes. Apart from the decrease in villi height and the corresponding increase in crypt depth, the villi architecture appears deformed. A decrease in villi height without a change in villi density or villi width would easily result in a decrease in the number of absorptive cells since it is only the mature cells on the upper part of the villi that have absorptive function. The corresponding increase in crypt depth does not enhance nutrient absorption since the undifferentiated cells in the crypt region are not important absorptive process.

Glutamine supplementation appears to prophylactically protect the intestinal mucosa against radiation damage (Klimberg et al., 1990b), to accelerate the healing of the small intestine following radiation injury

(Klimberg et al., 1990a) and in the present study protect the intestinal mucosa from the damaging effects of bacterial endotoxin. The mechanism of action by which glutamine maintains the normal villi architecture is not understood. However, it is conceivable that glutamine is involved in the replacement of lost cells or in the repair of damaged tissues. This proposition is based on the importance of glutamine for nucleic acid synthesis which is required for production of new cells.

Fig. V-1: Effect of endotoxin and glutamine supplementation on body weight

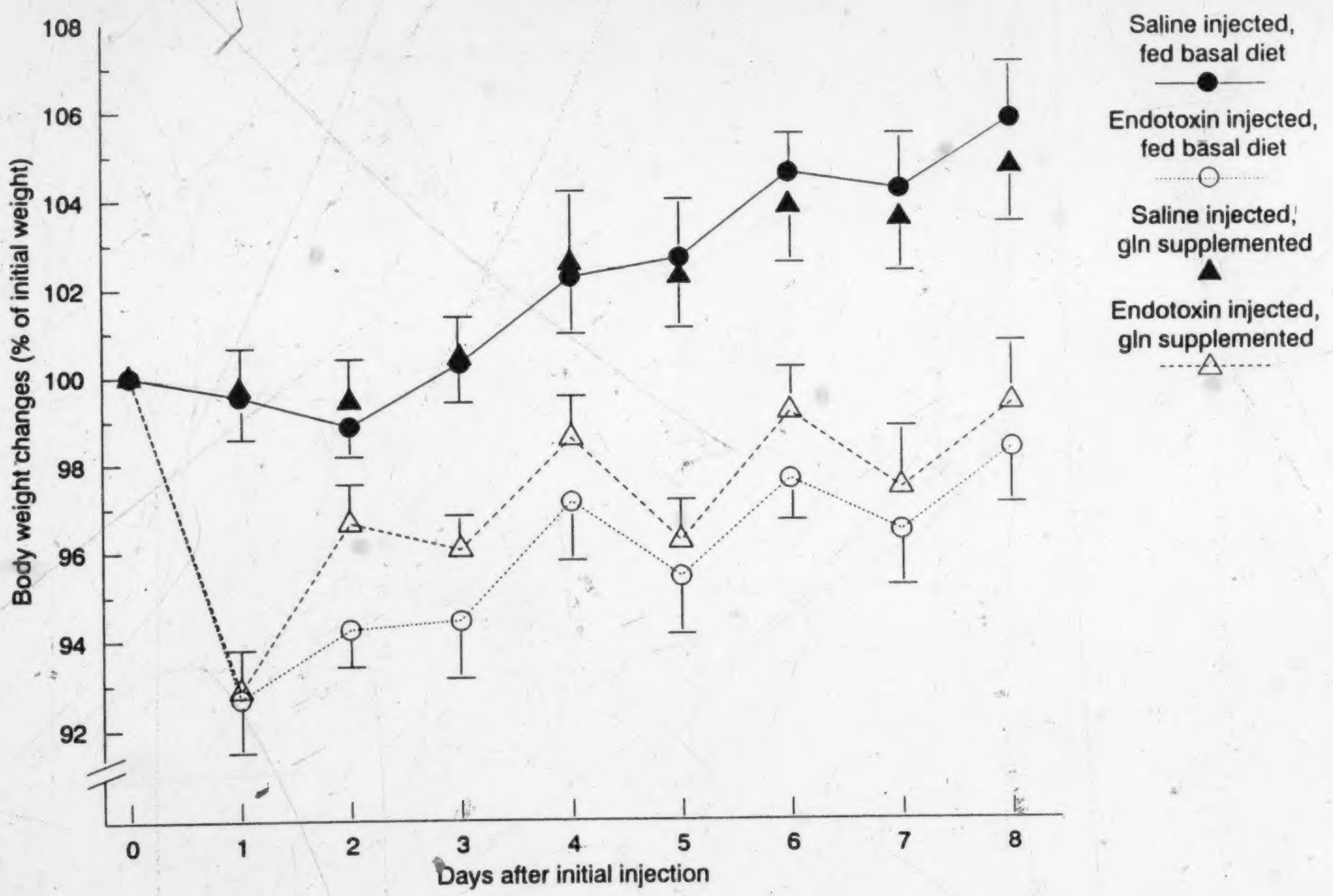


**Fig. V-2: Effect of endotoxin and glutamine supplementation on food intake**

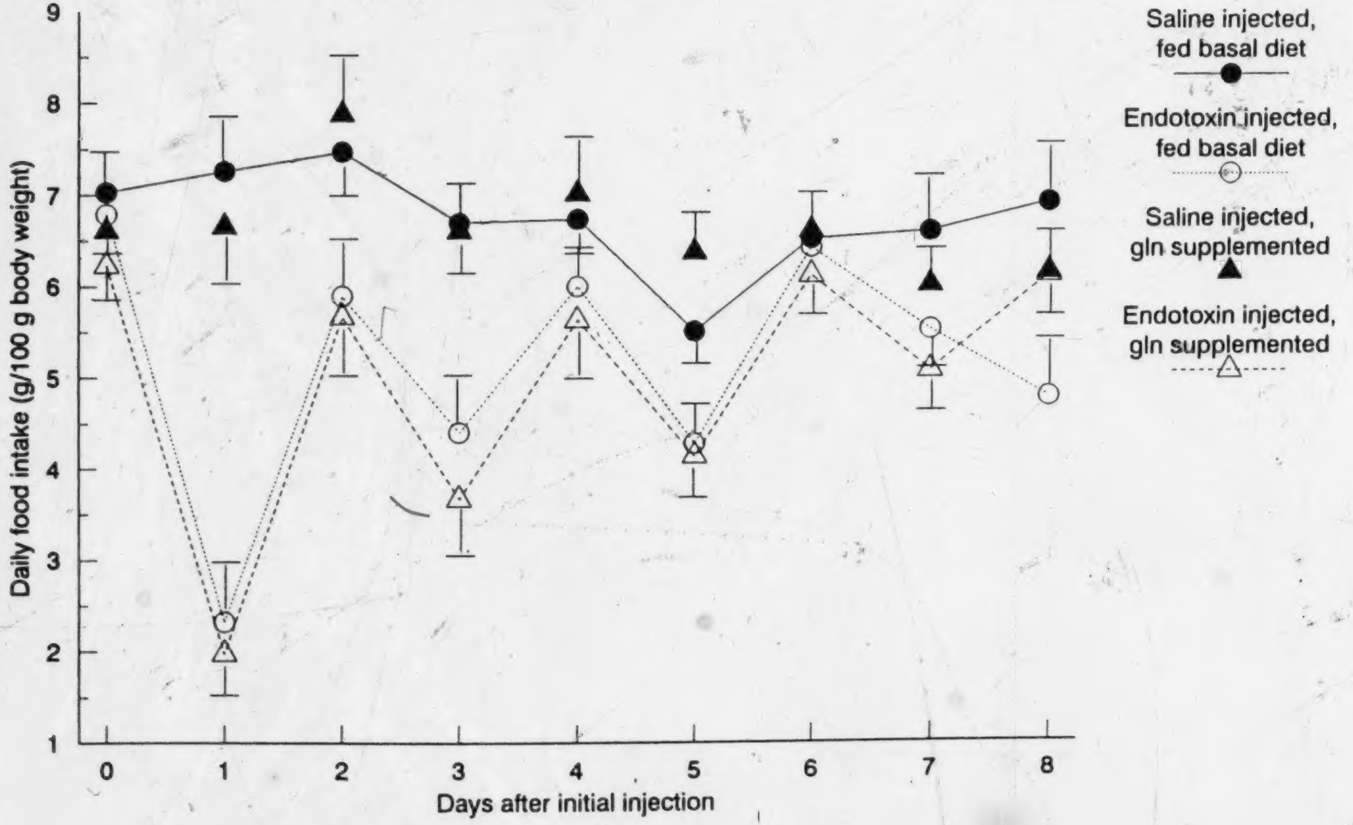


Table V-1: Amino Acid Composition of the Rat Chow

| Amino Acid | Concentration | |
|---|---|---|
| | mmol/100 g | g/100 g |
| Aspartic acid | 5.97 | 0.795 |
| Asparagine | 4.69 | 0.619 |
| Glutamic acid | 14.01 | 2.060 |
| Glutamine | 6.09 | 0.890 |
| Serine | 7.35 | 0.773 |
| Histidine | 2.94 | 0.456 |
| Glycine | 12.25 | 0.920 |
| Threonine | 5.99 | 0.714 |
| Arginine | 6.18 | 1.077 |
| Alanine | 14.13 | 1.259 |
| Tyrosine | 0.30 | 0.055 |
| Tryptophan | 3.09 | 0.630 |
| Methionine | 1.01 | 0.151 |
| Valine | 6.70 | 0.785 |
| Isoleucine | 4.73 | 0.781 |
| Phenylalanine | 5.02 | 0.658 |
| Leucine | 9.62 | 1.262 |
| Lysine | 8.43 | 1.232 |
| Cysteine | -[a] | |
| Proline | -[a] | |

[a]Not determined.

Table V-2: Effect of Dietary Glutamine Supplementation on the Response of Plasma Glutamine Concentration to the first Endotoxin Injection

About 500 ul of blood were collected from the tail before injecting the rats with endotoxin (400 µg/kg body weight). Blood was also collected from the tail at 24 and 48 h postinjection. Plasma obtained from the blood samples was analysed for glutamine. Values are presented as means ± SEM and values bearing different superscripts are significantly ($P<0.05$) different from each other. n=6

| Treatment | Glutamine concentration (nmol/ml)<br>Pre-injection | 24 h | 48 h |
|---|---|---|---|
| Basal diet | $915.8 \pm 68.1^a$ | $751.8 \pm 46.2^b$ | $825.9 \pm 12.7^a$ |
| Glutamine supplemented | $858.4 \pm 30.8^a$ | $756.0 \pm 19.1^b$ | $827.6 \pm 20.3^a$ |

Table V-3: Effect of Endotoxin and Dietary Glutamine Supplementation on Plasma Glutamine Concentrations at 192 h

Rats were injected with endotoxin (400 μg/kg body weight) at 0, 48, 96 and 144 h and then sacrificed at 192 h. Plasma samples were collected and analysed for glutamine. Control rats were fed a diet unsupplemented with glutamine and were injected with endotoxin-free saline. Values are presented as means ± SEM and values bearing different superscripts are significantly ($P \leq 0.05$) different from each other. n=6

| Treatment | Glutamine Concentration (nmol/ml) |
|---|---|
| **Saline injected:** | |
| basal diet | $862.9 \pm 48.7^a$ |
| glutamine supplemented diet | $872.0 \pm 51.8^a$ |
| **Endotoxin injected:** | |
| basal diet | $744.9 \pm 35.6^b$ |
| glutamine supplemented diet | $833.0 \pm 33.2^a$ |

Table V-4. Effect of Dietary Glutamine Supplementation and Endotoxin injection on Intramuscular Free Glutamine Concentration

Rats were injected with endotoxin (400 µg/kg body weight) at 0, 48, 96 and 144 h and then sacrificed at 192 h. The gastrocnemius and soleus muscles were analysed for intramuscular free glutamine. Control rats were fed a diet unsupplemented with glutamine and were injected with endotoxin-free saline. Values are presented as means ± SEM and values in the same column bearing different superscripts are significantly ($P<0.05$) different from each other. n=6

| Treatment | Gastrocnemius (nmol/mg) | Soleus (nmol/mg) |
|---|---|---|
| **Saline injected:** | | |
| basal diet | $3.07 \pm 0.14^a$ | $8.67 \pm 0.22^a$ |
| glutamine supplemented diet | $3.04 \pm 0.13^a$ | $8.04 \pm 0.38^a$ |
| **Endotoxin injected:** | | |
| basal diet | $2.60 \pm 0.09^b$ | $7.90 \pm 0.37^a$ |
| glutamine supplemented diet | $3.14 \pm 0.24^a$ | $8.56 \pm 0.31^a$ |

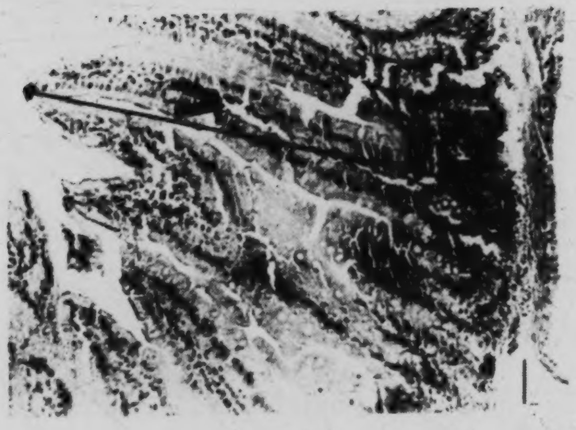
Table V-5: Effect of Glutamine Supplementation on Jejunal Mucosa Histology Following endotoxin Injection

Rats were injected with endotoxin (400 µg/kg body weight) at 0, 48, 96 and 144 h and then sacrificed at 192 h. Small segments of the jejunum were obtained for histological study. Control rats were fed a diet unsupplemented with glutamine and were injected with endotoxin-free saline. Values are presented as means ± SEM and values in the same column bearing different superscripts are significantly ($P \leq 0.05$) different from each other. n=6 rats

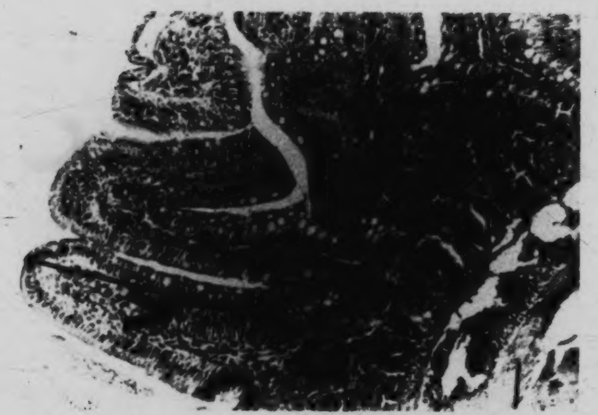
| Treatment | Crypt depth µm | Villi height µm | villi/crypt |
|---|---|---|---|
| Saline injected: | | | |
| basal diet | $162 \pm 3^{a}$ | $450 \pm 13^{a}$ | 2.78 |
| glutamine supplemented diet | $165 \pm 4^{a}$ | $467 \pm 10^{a}$ | 2.83 |
| Endotoxin injected: | | | |
| basal diet | $266 \pm 7^{b}$ | $300 \pm 10^{b}$ | 1.13 |
| glutamine supplemented diet | $170 \pm 5^{a}$ | $441 \pm 13^{a}$ | 2.59 |

Plate V-1. Microscopic Appearance of Paraffin-Embedded, Haematoxylin and Eosine Stained Jejunal Mucosa of Rats that were (a) Saline Injected and Fed Basal Control Diet; (b) Endotoxin Treated and Fed Basal control Diet; (c) Endotoxin Treated and Fed Glutamine Supplemented Diet. The Jejunal Mucosa Obtained from Rats which did not Receive Endotoxin, but were Fed Glutamine Supplemented Diet, did not Differ from Control Tissue Shown in (a). There were 6 rats per group.

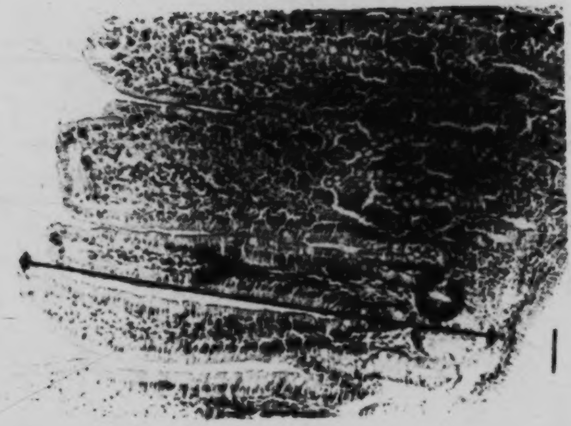
V=Villi, C=Crypt Depth, Bar=50 μm.



V-1a



V-1b



V-1c

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## VI General Discussion and Conclusions

**General Discussion:** Bacterial infections or toxins pose difficult challenges to human health and animal production. In the diseased state resulting from infections or toxin ingestion, potentially fatal physiologic and metabolic derangements can occur (Starnes et al., 1990). These may be manifested clinically as suboptimal performance by the organs or organ systems affected or organ failure. Also, productivity losses due to infection are quite substantial in the agricultural industry. Production losses arise from death and from retarded growth leading to late maturity and smaller live weight at maturity resulting in reduced efficiency of feed utilization. Food energy wasting could occur in two ways: (1) reduced efficiency of intestinal absorption of digested food, (2) increased energy required for maintaining elevated body temperature which is a common sequelae of infection-induced fever (Loew, 1974). The energy expenditure of humans, for example, increases by an additional 7.2% for each additional degree of Fahrenheit temperature (Howard, 1972) or (13% for a rise of 1 °C). This can induce secondary malnutrition in young animals (Howard, 1972).

Inhibition of gastric acid secretion during pyrexia induced by purified extracts from *Pseudomonas aeruginosa* was observed in dogs during the early studies of Blickenstaff and Grossman (1950). This observation was confirmed in rats by other researchers a few years later (Brodie and Kundrats, 1964). It is therefore quite possible that even the digestive process itself might be adversely affected during endotoxemia. A septic episode is not merely limited to the bacterial insult but has a profound effect on intermediary metabolism in several tissues (Severson et al., 1974; Hinshaw, 1976). In particular, alterations in carbohydrate metabolism have been implicated as playing important roles in the clinical outcome of sepsis (Holtzman et al., 1974). Such alterations include depletion of glycogen reserves, hexose phosphate and phosphoenolpyruvate concentrations and an elevation of the lactate:pyruvate ratio.

In the present study, endotoxin had no effect on apparent transport and metabolism of glucose at low glucose concentrations ranging from 0.15 - 1.0 mM but at higher concentrations, it caused a decrease in glucose transport. It is proposed that endotoxin reduces glucose transport by modifying glucose transporters thereby rendering them functionally ineffective. Therefore, endotoxin had no effect on glucose transport at low glucose concentrations possibly because there were enough functional glucose transporters (not affected by the endotoxin) to transport glucose at a rate that is similar to the control intestine. This proposal is supported by the lack of effect of endotoxin on the $K_M$ for glucose transport while the apparent $V_{max}$ was reduced. At high glucose concentrations there may not be a sufficient number of functional glucose transporters to transport glucose at a rate similar to that observed in the control intestine.

In mammalian tissues, transport of glucose into the cell is accomplished by carrier proteins located in the plasma membrane. Glucose would otherwise not readily cross the plasma membrane because of its impermeability to polar molecules. Two glucose carriers or transporters have been described in mammalian cells: the $Na^+$-glucose cotransporter and the facilitative glucose transporter (Baly and Horuk, 1988). The $Na^+$-glucose cotransporter or symporter is present in epithelial cells of the small intestine and proximal tubules of the kidney (Bell et al., 1990). This protein is found in the microvillus membrane and it actively transports glucose from the lumen of the small intestine or nephron against its concentration gradient by coupling glucose uptake with $Na^+$ which is being transported down its concentration gradient. The $Na^+$ gradient is maintained by the active transport of $Na^+$ across the basolateral membrane by the $Na^+$-$K^+$ ATPase. Absorbed glucose exits the intestinal absorptive cells by a $Na^+$-independent glucose transporter located in the basolateral membrane. The transport of glucose across the microvillus membrane appears to be the rate-limiting step in intestinal glucose absorption (Meddings and Westergaard, 1989).

The facilitative glucose transporters on the other hand are integral membrane proteins present on the surface of all cells (Bell et al., 1990). They transport glucose down its concentration gradient by energy-independent facilitative diffusion across the lipid bilayer. The facilitative glucose transporters function primarily in delivering glucose to the cells down its concentration gradient but can also transport glucose out of the cells, for example in the liver. This feature allows the liver to regulate blood glucose concentrations.

It is not very clear, which of the glucose transporters endotoxin is affecting. However the finding that phlorizin reduced the $I_{sc}$ in both the control and the endotoxin treated intestines by similar amounts, (the relative difference between the $I_{sc}$ in control and endotoxin treated intestines remained unaltered), leaves room to speculate that endotoxin may be acting on the facilitative glucose transporter or the $Na^+$-independent glucose transporter in the basolateral membrane. The tight junctions are also possible sites that endotoxin could affect intestinal glucose transport. It has been suggested that the tight juctions are not static gaskets as they are often assumed to be but may be viewed as relatively impermeable dynamic structures in which discontinuities, channels or pores reside (Madara, 1989; Madara, 1990). These pores may open and close like classical biomembrane channels.

The finding that most of the glucose taken up by flat sheets of the intestine at a concentration of 0.15 mM glucose was metabolised is indicative of the fact that the tissue requires most of the glucose provided at this concentration as an energy source when other sources such as glutamine are absent. This fuel is required for providing ATP for ion pumping and other energy requiring processes necessary for the survival of a living cell. It is not clear, to what extent other fuel sources for the small intestine, for example glutamine and ketone bodies (Newsholme and Leech, 1985a) are used for this purpose. However, as shown in chapter III enterocytes incubated with 5 mM glutamine plus or minus 10 mM glucose produced similar amounts of glutamate which can easily be converted to α-ketoglutarate, a TCA intermediate that can be metabolised to carbon dioxide with the release of energy. In the absence of glucose, glutamine may likely provide the energy required by the transporting enterocytes.

It has been reported by Parsons (1979) and Newsholme and Leech (1985a) that almost all the energy for the small intestine is provided by oxidation of glutamine and ketone bodies during starvation when luminal and plasma glucose concentrations are very low. In addition, as shown in the present study, at glucose concentrations ranging from 2.5 to 6.0 mM, a higher proportion of transported glucose carbon was metabolised to carbon dioxide by the endotoxin treated intestine than by the control intestine. This observation implies that transport and other basal processes that occur in living cells become energetically more costly, or less efficient during endotoxemia. A 2.5 fold increase in the active form of hepatic pyruvate dehydrogenase complex in response to sterile inflammation and small abscesses has been reported in rats by Vary et al. (1986). A more active pyruvate dehydrogenase may result in more pyruvate entering the TCA cycle as acetyl CoA. However, with large abscesses, there was a decrease in the active form of pyruvate dehydrogenase favouring glucose recycling.

The presence of endotoxin in tissues triggers activation of immunocytes resulting in the production of lymphokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1). IL-1-induced increases in glucose utilization in rat tissues rich in mononuclear phagocytes has been reported (Lang and Dobrescu, 1989). On the other hand, endotoxin-induced increases in regional glucose utilization by the small intestine of the rat has been reported to be TNF-independent (Lang et al., 1991). In the present report, it is not possible to delineate clearly the site of alterations in glucose utilization since various cell types including, mucosa cells, submucosa cells and the immunocytes are all present in the flat sheet of intestine used. There were decreases ($P<0.05$) in aspartic acid, glutamic acid, serine and threonine transport in the presence of endotoxin in the present study (see chapter II). Aspartic acid and glutamic acid are dicarboxylic amino acids while serine and threonine are hydroxylated amino acids. The similarity in functional groups of the amino acids affected suggests that endotoxin may have some effect on their transmembrane carrier systems. Possible effects of endotoxin on membrane carrier proteins has already been discussed.

The amide group of glutamine provides two of the nitrogen atoms required for purine synthesis, and one of the nitrogen atoms for pyrimidine nucleotide synthesis (Newsholme and Leech, 1985b). Glutamine is required by rapidly dividing cells (Eagle, 1955; Krebs, 1980; Newsholme et al., 1985) and is extensively oxidized by enterocytes (Newsholme and Parry-Billings, 1990), reticulocytes (Rapoport et al., 1971), lymphocytes (Ardawi and Newsholme, 1983; Newsholme and Parry-Billings, 1990), macrophages (Newsholme et al., 1987; Newsholme and Parry-Billings, 1990) and some tumour cells (Lanks, 1987; Rivera et al., 1988). Glutamine is not just extensively oxidized by enterocytes, but it is a major fuel for these cells (Windmueller and Spaeth, 1978; Nagy and Kretchmer, 1986; Watford et al., 1987). The dual role of glutamine in providing a substantial part of the energy requirements of the enterocytes and in providing amide nitrogen for purine and pyrimidine biosynthesis may explain why glutamine is required in large amounts by enterocytes.

Alterations in gastrointestinal tract metabolism may be exhibited when the types of energy substrates in the incubation medium are altered, for example by providing glutamine alone, glucose alone or glucose plus glutamine. Chapter III reports the effect of endotoxin on amino acid release and protein degradation in isolated enterocytes as well as glutamine metabolism in the presence or absence of glucose. Endotoxin increased protein degradation *in vitro* by the enterocytes. This observation when taken together with increased [$^3$H]thymidine incorporation into enterocyte DNA in response to endotoxin treatment *in vivo* reported in chapter IV, would lead to a suggestion that the increase in enterocyte apparent turnover rate is an adaptive response to the catabolic state induced by endotoxin.

It was surprising to find that tyrosine release into the incubation medium was elevated when enterocytes were incubated in the presence of 5 mM glutamine. An increase in the appearance of tyrosine in the incubation medium could be accounted for by: (1) increased rate of protein breakdown, (2) increased hydroxylation of phenylalanine to tyrosine, (3) increased efflux of tyrosine and other amino acids out of the enterocytes. Measurement of protein degradation via the release of [$^3$H]phenylalanine from prelabelled protein showed that glutamine itself did not increase protein breakdown as estimated by this approach. Also, in the presence of plasma concentrations of amino acids, tyrosine release increased in response to 5 mM glutamine whereas the net release of essential amino acids did not. Thus the increase in net tyrosine release may not be as a result of increased protein breakdown. Glutamine-induced hydroxylation of [$^3$H]phenylalanine could not be demonstrated qualitatively *in vitro* using a protocol that had been validated with hepatocyte homogenates. It was also demonstrated that glutamine did not affect the rate of [$^3$H]phenylalanine hydroxylation in hepatic tissue (see appendix 4). It would appear that there may be an enhanced efflux of selected amino acids at this relatively high concentration of glutamine.

One amino acid that is not a constituent of normal diets but was found in enterocytes was taurine. The exact function of taurine in the enterocytes is not clear but its involvement in numerous biological activities is beginning to unfold. Taurine functions as a conjugator of bile acids in the liver, a stabilizer of neural membranes, a vital amino acid for retinal function (Zelikovic et al., 1989), and a regulator of cell volume and osmolarity (Chesney, 1985). Since enterocytes are consistently faced with changing concentrations of electrolytes and nutrients in the intestinal lumen, taurine might function in the regulation of cell volume and osmolarity. In addition, the enterocyte taurine pool might serve as a dispensable taurine pool that could be readily mobilized to the liver for conjugation with bile acids.

The effects of endotoxin on food intake, intestinal disaccharidase activity and [$^3$H]thymidine incorporation into DNA were quite substantial (chapter IV). The most dramatic effect of endotoxin on food intake occured during the initial 24 h following the first injection. This drop in feed intake recorded in response to the first challenge with endotoxin was followed by substantial amelioration in the response to subsequent challenges. The disaccharidase activities except isomaltase were quite sensitive to the initial drastic effects of endotoxin but the adaptive food intake recovery was much faster than the recovery in enzyme activity. It would appear that endotoxin interacts with specific membrane components (Shrands, 1973) to bring about alterations in some biochemical properties of the cell. Complete recovery in such properties may only be achieved after 3 - 6 days following withdrawal, a time period during which the enterocytes would have had adequate time to completely turnover.

Dietary glutamine supplementation plays a promising role in alleviating some of the responses that characterise endotoxemia (see chapter V). Rats injected with endotoxin and fed the basal diet had a significantly ($P<0.01$) lower villi height and higher crypt depth compared to the saline injected group fed the basal diet. Glutamine supplementation preserved the normal features of the mucosa during endotoxin administration. Glutamine supplementation in the absence of endotoxin had no effect on either villi height or crypt depth in control animals. The mechanism by which dietary glutamine supplementation protects the normal villi architecture is not known. However, the increase in thymidine incorporation during endotoxemia suggests that there is an increased demand for nucleotide biosynthesis. The ameliorating role of glutamine may be linked with the provision of nitrogen for nucleotide biosynthesis required for the replacement of the enterocytes sloughing off from the villi surface. Secondly, activation of the immune system in response to endotoxin increases the demands for glutamine (see Fig. I-2). Where the additional need for glutamine is not met the immune system will function at a suboptimal level. The resulting inefficient defences and repairs could then lead to defects in the structure of the villi. The response of the immune system to, for example, an invasion by a microorganism must be rapid; hence glutamine availability and the rate of utilization must always be rapid and extensive in order to provide optimal conditions for immediate response to an immune challenge. This in part explains why the cells of the small intestine consume large amounts of glutamine (Newsholme and Parry-Billings, 1990). The small intestine is exposed to numerous biological insults and therefore must always have an adequate readily available supply of glutamine to activate the immune cells very rapidly and optimally. The large uptake of glutamine is an insurance against the immune system being caught off guard.

**Conclusions:** The following conclusions may be drawn from the materials presented in this thesis:- In response to bacterial endotoxin in the rat

1. there were decreases in apparent transport of glucose, aspartic acid, glutamic acid, serine and threonine by the ileum,
2. the ileum metabolized a higher proportion of the glucose that it transports,
3. the enterocytes dramatically increased their rate of protein degradation,
4. there was a reduction in both food intake and enterocyte disaccharidase activities,
5. there was an increase in apparent enterocyte turnover,
6. plasma and intramuscular glutamine concentrations decreased,
7. villi architecture of the small intestine was altered following 192 h of treatment, and dietary glutamine supplementation prevented these

changes in villi architecture and also prevented the drop in plasma and muscle glutamine concentrations.

**Suggestions for Further Studies**: During the course of this research, a number of interesting biological questions for further research became evident. These include:

1. Purification and adequate characterization of endotoxin receptors. Once the properties of the receptor(s) are known, it may be possible to develop possible blockers that can prevent them from accepting endotoxin. Since the effect of endotoxin is most drastic during the first few hours to 1 day following challenge, blockers of endotoxin receptors could help the animal survive this crucial stage (particularly young animals).
2. Endotoxin was found in this study to increase protein breakdown in enterocytes. The mechanism of protein degradation in enterocytes remains to be characterized.
3. Endotoxin increased thymidine incorporation rate, suggesting increased cell production rate. Further studies should be carried out on the effect of endotoxin on enterocyte cycle time, growth fraction and migration rates so as to determine precisely if endotoxin increases enterocyte turnover rate.
4. The time required for enterocyte disaccharidase activity to return to normal after the adaptation in terms of food intake has been accomplished is not clear and requires further

characterization.

5. Glutamine had no significant effect on enterocyte protein breakdown *in vitro*. The mechanism by which the presence of glutamine increased tyrosine release requires further elucidation.
6. The ameliorating role of glutamine on the endotoxic response of the gastrointestinal tract reported in this study needs further exploration. Damage to gut structure in response to bacterial toxins or other xenobiotics may be immune-mediated and therefore the action of glutamine is via its influence on immunocytes such as lymphocytes, monocytes and macrophages.
7. It needs to be determined if the benefits of glutamine supplementation are sufficient to warrant mandatory addition of glutamine to diets in certain clinical states.

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# VII Appendices

## Appendix 1: Composition of Krebs-Ringer Bicarbonate Buffer (KRB)[1]

| Reagent | Stock Solution (g/l) | 1x (ml) | 2x (ml) | 5x (ml) | 10x (ml) | 20x (ml) | Final Concentration (mM) |
|---|---|---|---|---|---|---|---|
| NaCl | 20 | 35 | 70 | 175 | 350 | 700 | 119 |
| $NaHCO_3$ | 7 | 30 | 60 | 150 | 300 | 600 | 25 |
| KCl | 36 | 1 | 2 | 5 | 10 | 20 | 4.82 |
| $CaCl_2.2H_2O$ | 1.47 | 1 | 2 | 5 | 10 | 20 | 1.0 |
| $MgSO_4.7H_2O$ | 30.8 | | | | | | |
| Or ($MgSO_4$) | 15.1 | 1 | 2 | 5 | 10 | 20 | 1.25 |
| $NaH_2PO_4.H_2O$ | 17.3 | | | | | | |
| Or ($NaH_2PO_4$) | 15.0 | 1 | 2 | 5 | 10 | 20 | 1.25 |
| HEPES (pH 7.4) | 9.54 | 5 | 10 | 25 | 50 | 100 | 2.0 |
| Water | | 26 | 52 | 130 | 260 | 520 | |
| Total Volume (ml) | | 100 | 200 | 500 | 1000 | 2000 | |

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[1] Supplemented with insulin (0.1 U/ml) and when appropriate, 10 mM glucose (Goldberg et al., 1975) and/or amino acids as described under Materials and Methods. Calcium enhances clumping of enterocytes (Kimmich, 1970), so $CaCl_2$ was absent from KRB used for enterocyte isolation and incubation. Note that HEPES/NaOH enhances the buffering capacity of KRB.

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## Appendix 2: Amino Acid Analysis by High Performance Liquid Chromatography

### Chromatographic System:

Separation and quantification of amino acids was accomplished with a Varian 5000 high performance liquid chromatograph and a Varian Fluorichrom detector (excitation 340nm emission 450nm). Samples were mixed 1:1 with a fluoraldehyde reagent prior to injection with a delay time of 12 sec. Uniform mixing of samples before sampling was achieved using a modified Technicon autosampler and a Chemlab peristaltic pump with a stainless steel mixing tee. The samples were injected using a Valco autoinjector valve equipped with a 20 µl loop. The analytical column used was a Supelcosil 3 micron LC-18 reverse phase column (4.6 x 150 mm; Supelco) equipped with a guard column (4.6 x 50 mm) packed with Supelco LC-18 reverse phase packing (20 - 40 µm). Chromatographic peaks were recorded using a Fisher recorder, and integration was accomplished using a Hewlett Packard 2645 data system with a Hewlett Packard 18652A A/D converter.

Buffer solutions and detailed mobile phase gradients employed for the various separations are provided below.

### Buffer Solutions:

Solvent A: 0.1 M sodium acetate, pH 7.2

Solvent B: methanol.

**Preparation of Fluoraldehyde Reagent**:

One half gram of o-phthaldialdehyde (OPA) was dissolved in 12.5 ml methanol and then 112 ml 0.04 M sodium borate buffer (pH 9.5) were added along with 0.50 ml 2-mercaptoethanol and 4 ml Brij 35.

**Standard Solutions**:

**Amino Acid Solution**: Two hundred and fifty µl of a commercial amino acid mixture (Sigma, 2.5 mM) plus 125 µl of 5 mM citrulline and taurine, and 125 µl of 5 mM glutamine, asparagine and tryptophan solutions were diluted to 25 ml. The resulting solution contained 25 nmol/ml of each amino acid.

**Ethanolamine**: 25 nmol/ml solution. Used as internal standard for determination of total amino acid profile of samples that contained no ethanolamine.

**Amino guanidopropionic acid (AGPA)**: 25 nmol/ml. Internal standard for glutamine determination.

**Sample Preparation**:

**Plasma Samples**: To 100 µl of sample or amino acid standard were added 100 µl of internal standard. Samples were vortexed and 400 µl of 5 % w/v trichloroacetic acid (TCA) were added and centrifuged at 3000 x g for 15 min. The supernatant was treated with 300 µl of saturated potassium borate and 500 $\mu$l of water.

**Incubation Media**: One hundred $\mu$l of sample or amino acid standard were treated with 100 $\mu$l of ethanolamine, 100 $\mu$l of 1.2% (w/v) benzoic acid, 100 $\mu$l of saturated potassium borate and 1.0 ml of water.

**Intracellular Samples**: A 100 $\mu$l aliquot of sample or amino acid standard in 2% TCA was treated with 100 $\mu$l of internal standard, 100 $\mu$l of saturated potassium borate and 1.10 ml of water.

**Feed Hydrolysates**: Hydrolysates were diluted 30 times and centrifuged at 2500 x g for 15 min. One hundred $\mu$l of the clear supernatant or amino acid standard were treated with 100 $\mu$l of ethanolamine, 100 $\mu$l of saturated potassium borate and 1.1 ml of water.

### Mobile Phase Gradient:

**Total Amino Acid Profile**: For plasma, incubation media and intracellular samples.

| Time (min) | %A | %B |
|---|---|---|
| 0.0 | 100 | 0 |
| 0.1 | 86 | 14 |
| 22.0 | 85 | 15 |
| 22.1 | 70 | 30 |
| 26.0 | 69 | 31 |
| 26.1 | 53 | 47 |
| 34.0 | 50 | 50 |
| 38.0 | 30 | 70 |
| 40.0 | 0 | 100 |
| 42.0 | 0 | 100 |
| 42.1 | 100 | 0 |

**Flow Rate**: 1.1 ml/min.

**Analysis Time**: 49 min.

**Total Amino Acid Profile**: For hydrolysates

| Time (min) | %A | %B |
|---|---|---|
| 0.0 | 100 | 0 |
| 0.1 | 85 | 15 |
| 14.0 | 80 | 20 |
| 18.0 | 68 | 32 |
| 18.1 | 55 | 45 |
| 24.1 | 50 | 50 |
| 28.1 | 30 | 70 |
| 30.0 | 0 | 100 |
| 32.0 | 0 | 100 |
| 33.0 | 100 | 0 |

**Flow Rate**: 1.1 ml/min.

**Analysis Time**: 39 min.

Glutamine:

| Time (min) | %A | %B |
|---|---|---|
| 0.0 | 100 | 0 |
| 0.1 | 86 | 14 |
| 10.0 | 85 | 15 |
| 16.0 | 85 | 15 |
| 16.1 | 0 | 100 |
| 20.0 | 0 | 100 |
| 20.1 | 100 | 0 |

**Flow Rate:** 1.1 ml/min.

**Analysis Time:** 24 min.

Phenylalanine:

| Time (min) | %A | %B |
|---|---|---|
| 0.0 | 62 | 38 |
| 6.0 | 50 | 50 |
| 6.1 | 20 | 80 |
| 6.9 | 20 | 80 |
| 7.0 | 62 | 38 |

**Flow Rate:** 1.1 ml/min.

**Analysis Time:** 11 min.

## Appendix 3-1: Measurement of Cell Viability

The enterocytes were isolated according to the method of Reiser and Christiansen, (1971) as modified by Watford et al., (1979). Viability testing was carried out immediately after harvesting the cells by two methods: the trypan blue exclusion method and the lactate dehydrogenase leakage method.

### Trypan Blue Exclusion Method:

**Principle**: Cells that are viable or have an intact plasma membrane are able to exclude trypan blue but those cells with damaged plasma membrane are unable to exclude the dye. Cells that completely exclude the trypan blue are considered viable and their number is expressed as a percentage of the total cell population.

**Procedure**: A pilot study was first carried out to establish the optimum trypan blue concentration that is required because different concentrations of trypan blue including 0.2 mM (Belinsky et al., 1984), 0.1% (Gibson et al., 1989), 0.2% (Lash and Tokarz, 1989) and 0.5% (Reiser and Christiansen, 1971) have been employed. The enterocytes have a tendency to aggregate and so it was much easier to estimate the number of enterocytes that were stained when a low concentration of trypan blue was used. The concentration of 0.2 mM gave the best result and was subsequently used.

The following procedure was finally adopted. The cells were incubated for 3 minutes in 0.2 mM trypan blue in phosphate buffered saline (pH 7.4) and examined by light microscopy. Both the number of cells that were able to exclude trypan blue (viable cells) as well as those that were stained with the dye (non viable cells) were counted. The viability was expressed as the number of cells that excluded trypan blue as a percentage of the total population of cells.

To determine the maximum length of time for which the cells remain viable for metabolic studies, the enterocytes were incubated for 0, 15, 30, 45, 60, 90, 120, minutes in oxygenated (95% $O_2$ : 5% $CO_2$) Krebs-Ringer bicarbonate buffer (KRB). Aliquots of the incubated cells were transferred into 0.2 mM trypan blue for 3 min and subsequently examined on the microscope.

**Lactate Dehydrogenase (LDH) Leakage**: Pelleted enterocytes were resuspended in saline (see isolation protocol) and 0.2 ml aliquots were incubated with 3.3 ml KRB for 10, 15 30 45 60 90 and 120 min with constant gassing. At the end of the incubation period, the incubation medium was centrifuged for 2 min at 1000 x g then the supernatant was assayed for LDH activity by following the coupled oxidation of NADH at 340 nm according to the method of Bergmeyer et al., (1963). Two hundred microliters of the original enterocyte suspension were ultrasonically lysed in 3.3 ml KRB and the LDH activity was measured to obtain the total LDH activity. The fraction of LDH activity specifically released during incubation was expressed as a percentage of the total LDH activity.

Comments: Even though Kimmich, (1970) has reported that enterocytes from chicken retained viability and produced carbon dioxide from glucose at a linear rate for at least 2 h, it was found in this study that up to 80% of the cells were no longer viable after 2 h. The LDH leakage method was found not to be as sensitive as the trypan blue uptake method since apparent viability according to the LDH assay after 90 min incubation was much higher than the value obtained by the trypan blue exclusion method. See earlier report by Blinsky et al., (1984).

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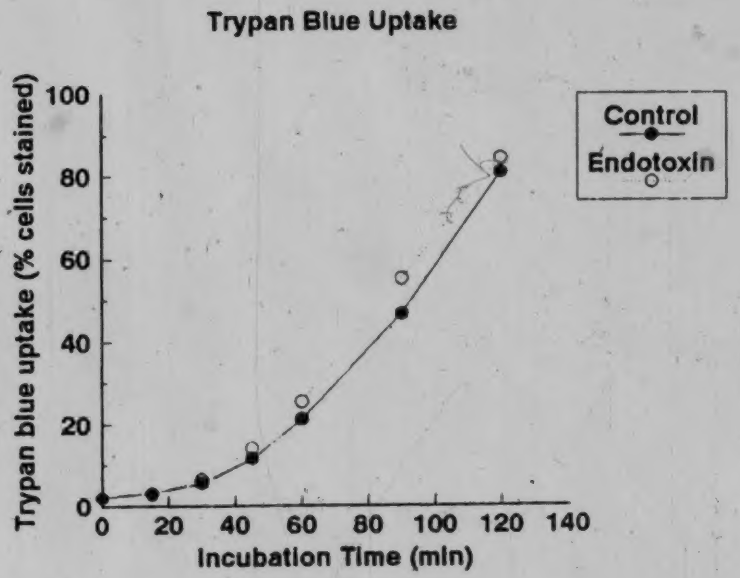
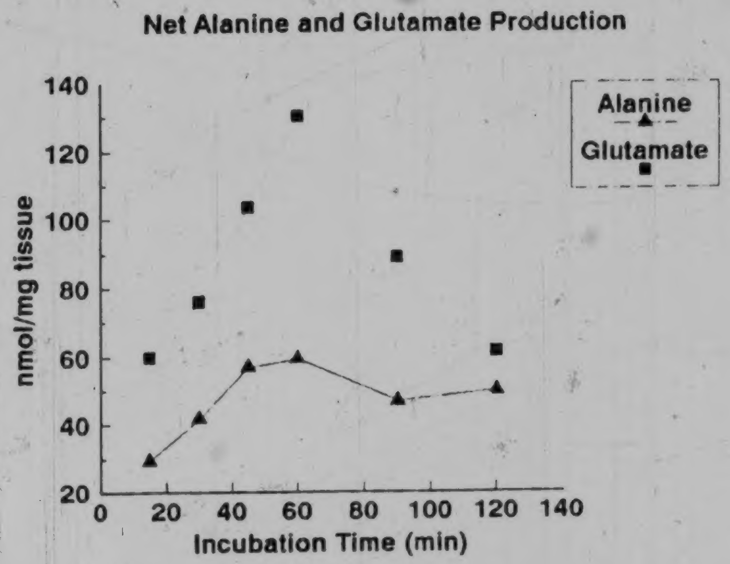
## Appendix 3-2. Enterocyte Viability Assessment


Trypan Blue Uptake


Net Alanine and Glutamate Production

Only viable cells are able to exclude the dye. Approximately 80% of the enterocytes remained viable during 1 h of incubation. Glutamate production remained linear during 1 h of incubation, while alanine production remained linear during 45 min of incubation.

## Appendix 4-1: Determination of Liver and Enterocyte Phenylalanine Hydroxylase

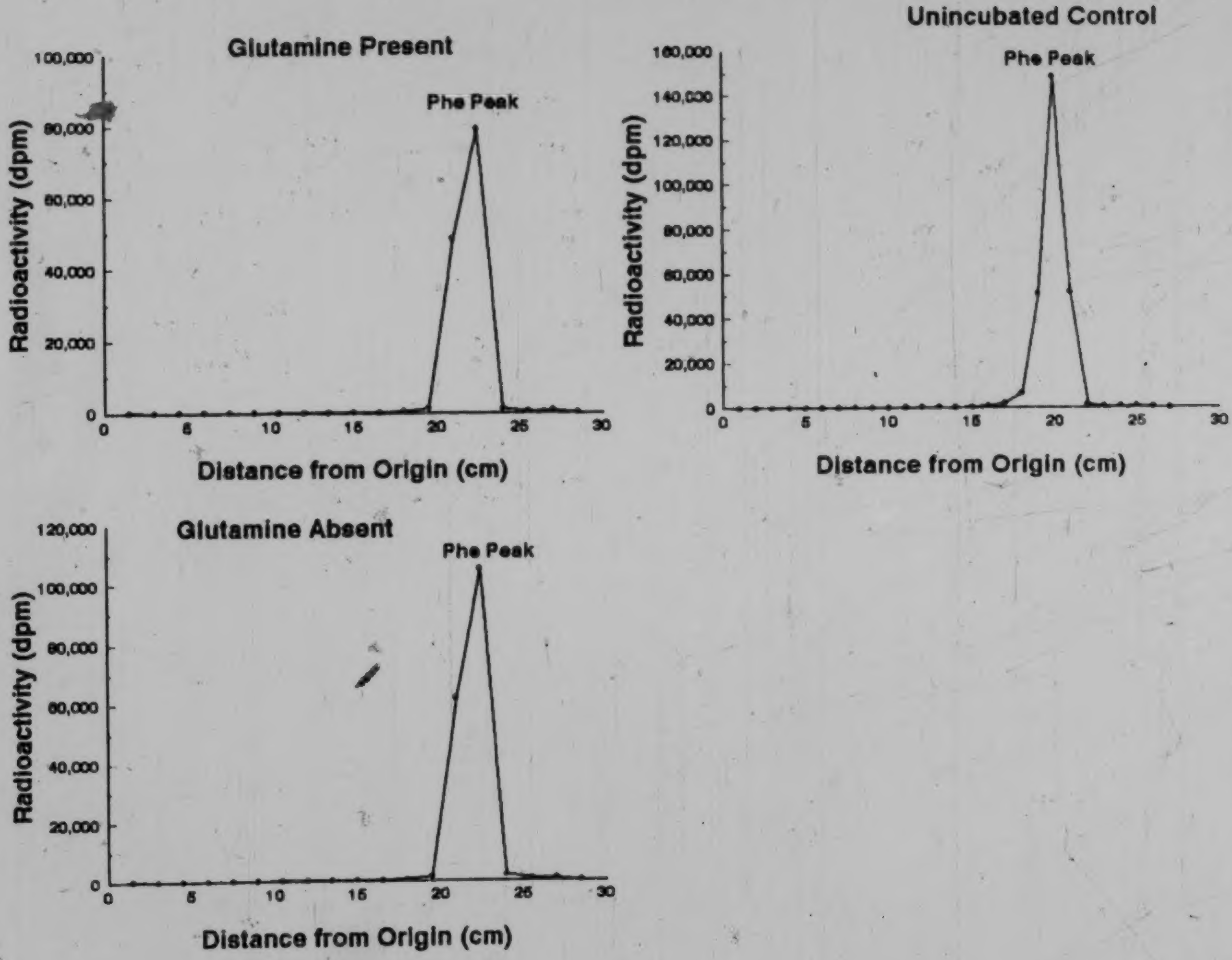
Phenylalanine hydroxylase activity was determined by the method of McGee et al., (1972). Freshly excised liver tissue or isolated enterocytes from 6 male Sprague Dawley rats were homogenized in 9 vol. of 0.15 M KCl and centrifuged at 16000 x g for 15 min. Two hundred $\mu$l of the supernatant were incubated with 10 mM [$^3$H]phenylalanine, 0.75 mM 6,7-dimethyl-5,6,7,8-tetrahydropterin and 5 mM dithiothreitol in a total volume of 1 ml in the presence or absence of glutamine. The reaction was initiated by addition of the supernatant. Immediately before the addition of the supernatant (unincubated tubes only) or after 20 min incubation at 37 °C, 1 ml of 12 (w/v) trichloroacetic acid was added to the reaction mixture to denature the enzyme. The contents were mixed and centrifuged for 20 min at 3000 x g. [$^3$H]tyrosine produced by the action of phenylalanine hydroxylase on [$^3$H]phenylalanine was separated from the [$^3$H]phenylalanine by paper chromatography in n-butanol: acetic acid: water (2:1:1), see appendixes 4-2&3. Non radioactive phenylalanine and tyrosine were also spotted as carriers and identification of the peaks was carried out by spraying half the chromatogram with 0.25% ninhydrin in acetone.

**Findings**: The [$^3$H]phenylalanine used was radiochemically pure as only one peak was present in the unincubated solution. Phenylalanine hydroxylase activity was not detected in the enterocytes. Qualitative demonstration of phenylalanine hydroxylase activity in the liver served to validate the assay method.

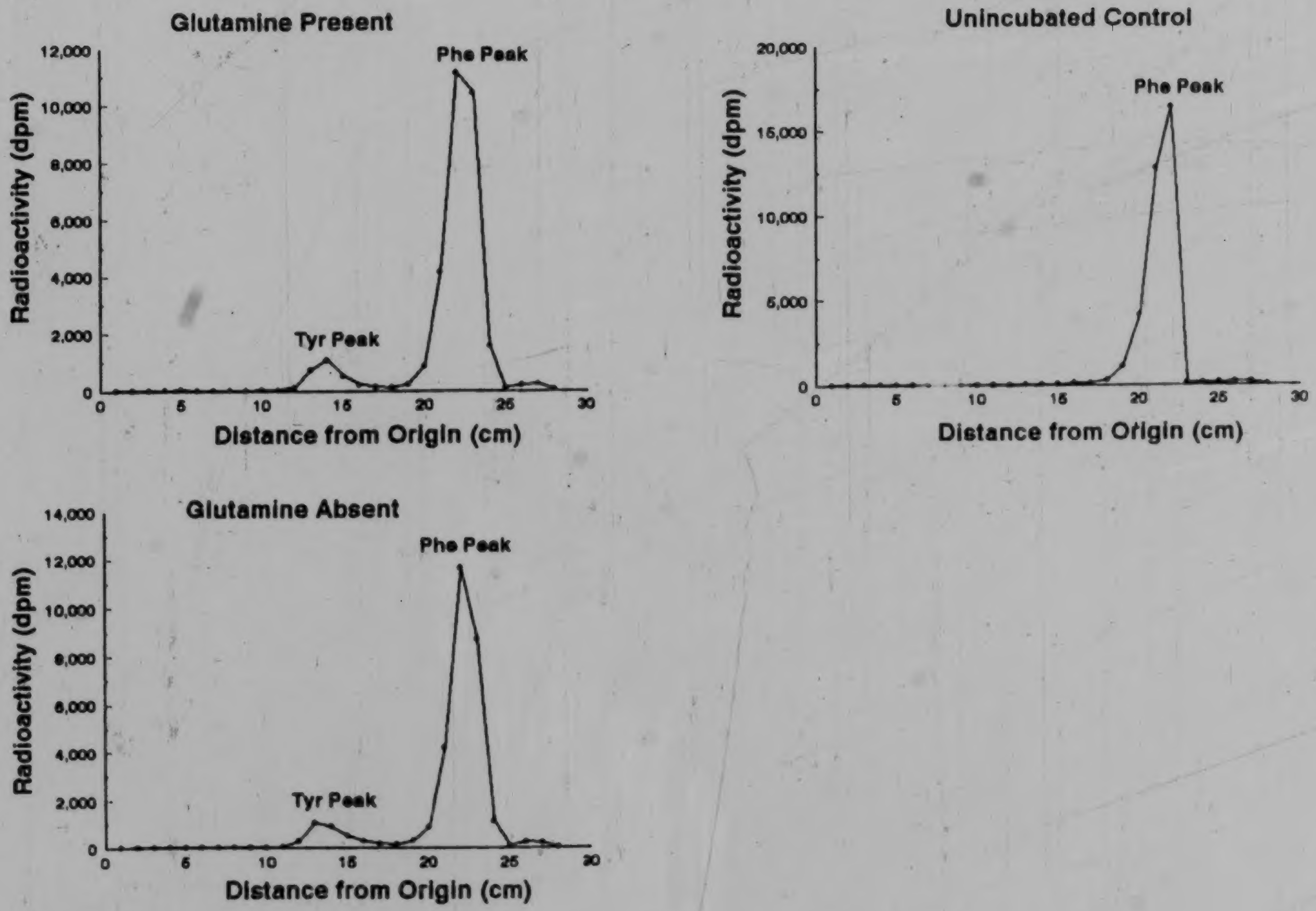
Reference:

McGee, M.M., Greengard, O. and Knox, W.E. (1972) The quantitative determination of phenylalanine hydroxylase in rat tissues. Biochem. J. **127**, 669-674.

Appendix 4-2. Phenylalanine Hydroxylase Activity in Enterocytes



Appendix 4-3. Phenylalanine Hydroxylase Activity in Hepatic Tissue



Glutamine Present

Unincubated Control

Glutamine Absent

## Appendix 5: Tissue Preparation for Histological Examination

**Reagents:**

A. **Buffered 4% formalin ( pH 7.0 ):**

| | |
|---|---|
| Formalin | 100 ml |
| Water | 900 ml |
| Sodium dihydrogen phosphate ( $NaH_2PO_4.H_2O$ ) | 4.0 g |
| Disodium hydrogen phosphate ( $Na_2HPO_4$ ) | 6.5 g |

B. **Harris' Haematoxylin:**

| | |
|---|---|
| Haematoxylin | 1.0 g |
| Absolute alcohol | 10.0 ml |
| Potassium alum | 20.0 g |
| Distilled water | 200.0 ml |
| Mercuric oxide | 0.5 g |

Haematoxylin and potassium alum were individually dissolved in 10 ml of alcohol and 200 ml of hot water, respectively and mixed. The mixture was quickly boiled and the mercuric oxide was added. The resulting dark purple solution was rapidly cooled under the tap and filtered. Eight ml of glacial acetic acid were then added to sharpen nuclear staining.

C. **Eosin:** A 5% aqueous stock solution of eosin was prepared. This was then diluted to 1% before use.

D. **Other reagents:**

| | |
|---|---|
| Ethanol | 50% |
| Ethanol | 70% |
| Ethanol | 80% |
| Ethanol | 80% |
| Ethanol | 95% |
| Ethanol | 100% |
| Ethanol | 100% |
| Xylene | |
| Xylene | |

1. **Fixation:** Fixation was carried out in **buffered formalin**. Small pieces of the intestinal segments ( 1.5 cm in length ) were cut out and placed in the 4% buffered formalin for 48 h. Four per cent buffered formalin imparts effective consistency without excessive hardening and is tolerant in terms of duration of fixation.

2. **Dehydration, Clearing and Paraffin Impregnation**: Dehydration was carried out in seven exchanges of alcohol solution and clearing was done in two exchanges of xylene. Paraffin impregnation was carried out in three exchanges of paraffin (see schedule below).

| | | |
|---|---|---|
| Ethanol | 50% | 1 h |
| Ethanol | 70% | 1 h |
| Ethanol | 80% | 1 h |
| Ethanol | 80% | 1 h |
| Ethanol | 95% | 1 h |
| Ethanol | 100% | 1 h |
| Ethanol | 100% | 1 h |
| Xylene | | 1 h |
| Xylene | | 1 h |
| paraffin (paraplasts) | | 1 h |
| paraffin | | 1 h |
| paraffin | | 1 h |

The purpose of paraffin wax impregnation was to remove the clearing agent and for the samples to be completely permeated by the paraffin wax which was subsequently allowed to harden to produce a block from which sections were cut. This step was carried out at 60 °C and cooled in ice.

**Sectioning**: Five micron (5 $\mu$m) sections were cut and transfered into a water bath at 45 °C. The sections were picked on glass slides which had been smeared with gelatine (0.1%).

**Dewaxing**: The following steps were adopted for the dewaxing process.

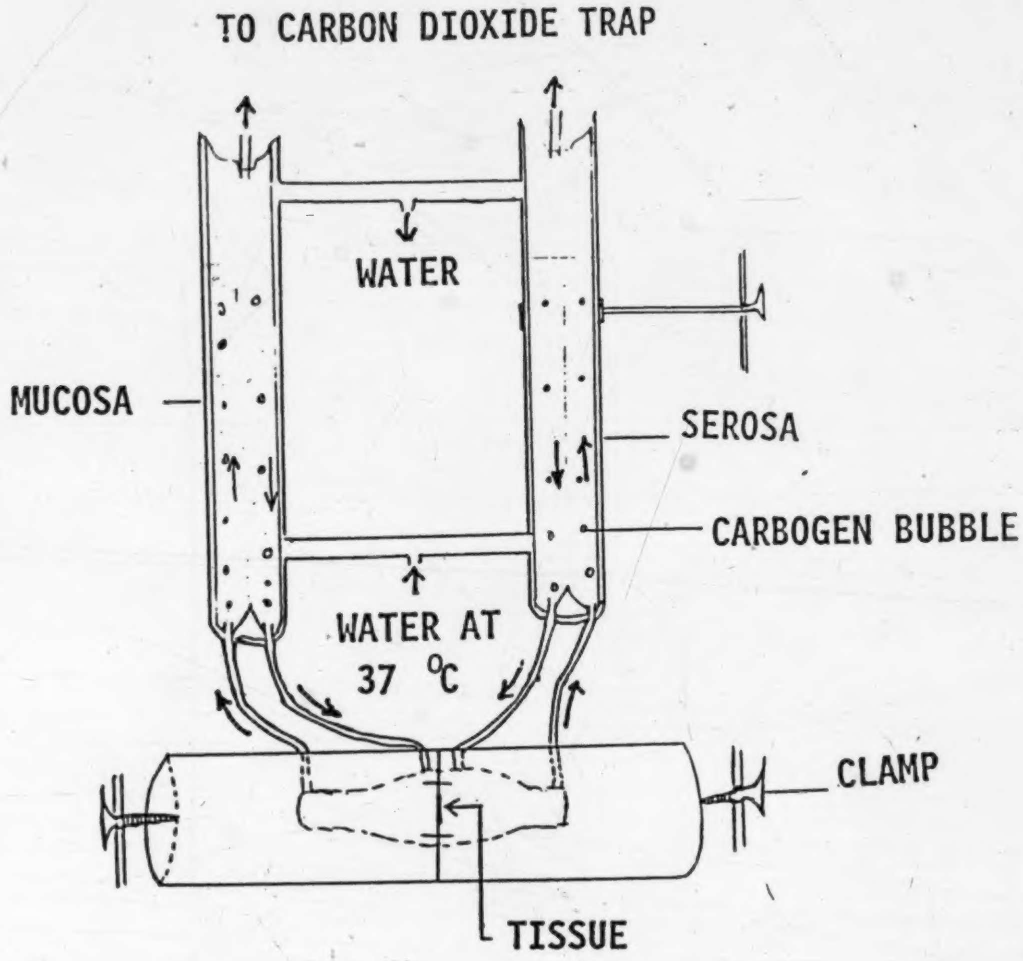
1. Four min in two exchanges of xylene.
2. One min in two exchanges of absolute ethanol.
3. One min in 80% ethanol.
4. One min in 50% ethanol.
5. One min in water.

**Staining**:

1. Ten min in haematoxylin.
2. Wash off excess dye.
3. Imerse in 1% HCl in 70% ethanol for 45 sec.
4. Wash thoroughly with tap water (slightly alkaline).
5. Three min in 1% eosin.
6. Wash with tap water.
7. One min in absolute ethanol.
8. One min in 50:50 ethanol-xylene.
9. One min in xylene.

**Mounting**: A drop of permount was placed on the slide and the cover slip was placed in position.

**Appendix 6**: Diagrammatic Representation of a Ussing Chamber Showing a Mounted Tissue and the Mucosal and Serosal Compartments.


TO CARBON DIOXIDE TRAP
WATER
MUCOSA
SEROSA
CARBOGEN BUBBLE
WATER AT
37 °C
CLAMP
TISSUE

USSING CHAMBER SHOWING MOUNTED TISSUE